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14. ABSTRACT
During the second year of the project, we completed all analyses for the pharmacokinetic study with gamma-tocotrienol (GT3) in four NHPs. Specifically, we completed subtask 3, 4, and 5 of major task 1 and 2. PK analysis of GT3 serum revealed results consistent with that with a previous PK study conducted in the PI's lab with GT3 of DTRA project. In addition, subtask 1 of major task 3 was completed during this reporting period. We collected and analyzed samples for complete blood counts, blood biochemistry, cytokine profile, citrulline, flow cytometric phenotyping of hematopoietic cells and gastrointestinal studies. We have completed one cohort of subtask 2 and anticipate to begin a second cohort of NHPs in order to complete subtask 2 in the very near future. Overall, project is progressing well.

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TABLE OF CONTENTS

1. INTRODUCTION:

Gamma-tocotrienol (GT3) is a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA), and has received great attention in recent years. Its antioxidant activity was the compelling reason to evaluate it for its radioprotective efficacy. GT3 protected almost 100% of mice against a lethal dose of ^{60}Co γ-radiation when administered subcutaneously (sc) 24 h before radiation exposure and its dose reduction factor (DRF) was 1.29. GT3 also accelerated hematopoietic recovery in irradiated mice compared to vehicle controls. GT3 has been shown to protect hematopoietic stem cells as well as reduce DNA damage. In the current proposal, the main focus is to (1) Investigate the radioprotective efficacy of GT3 in NHPs using different doses of radiation for whole body exposure. As stated above, in a pilot study using a small number of NHPs, GT3 demonstrated excellent radioprotective efficacy when administered 24 h before whole body ^{60}Co γ-radiation exposure. (2) Study hematopoietic and gastrointestinal injury, accelerated recovery, and efficacy biomarkers in NHPs treated with GT3. Hematopoietic and gastrointestinal injury as well as accelerated recovery by GT3 will be studied using blood, bone marrow, peritoneal aorta, and gastrointestinal tissue obtained from animals exposed to whole-body and partial-body radiation exposures. Vascular injury will also be studied since the radioprotective efficacy of GT3 is related to its properties as an HMG-CoA reductase inhibitor. The above studies will form the foundation for clinical trials for safety in humans, heading towards licensure from the US FDA. With the deployment of GT3, forces exposed to moderate to high doses of ionizing radiation will demonstrate enhanced survivability, expanding the range of operable threat environments and options available to our military. Our study will advance development toward 'field use' of GT3 and inclusion in the Strategic National Stockpile.

2. KEYWORDS:

Biomarkers, cytokines, endothelial cells, growth factors, hematopoietic and gastrointestinal sub-syndromes, gamma-tocotrienol, pharmacokinetics, radiation countermeasure, radioprotector, tocol, vitamin E

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: Investigate GT3 obtained from a new vendor for pharmacokinetics (PK) and skin irritation Test of new product for skin irritation/PK: 4 NHPs	**Timeline (Months)**	**Progress**	**Site 1** Prof. Vijay K. Singh AFRRI	**Site 2** Prof. Martin Hauer-Jensen UAMS
Major Task 1 and 2: Evaluation of GT3 from new vendor for PK and skin irritation	Months			
Subtask 3. PK of new formulation: Analysis for CBC, blood biochemistry, cytokine profile	10-15	Completed	Dr. V. K. Singh	
Subtask 4. Flow cytometric phenotyping of hematopoietic cells	10-15	Completed	Dr. V. K. Singh	Prof. Hauer-Jensen
Subtask 5. Hematopoietic and stem cell studies, gastrointestinal studies, vascular/endothelial studies	10-15	Completed	Dr. V.K. Singh	Prof. Hauer-Jensen
Milestone Achieved: AFRRI IACUC/ACURO Approval, PK of GT3 obtained from new vendor	15		Dr. V. K. Singh	Prof. Hauer-Jensen

Specific Aim 2: Investigate the radioprotective efficacy of GT3 in NHPs using different doses of whole body radiation exposure: Vehicle 5.8 Gy – 16 NHPs GT3 5.8 Gy – 16 NHPs Vehicle 6.5 Gy – 16 NHPs GT3 6.5 Gy – 16 NHPs Total 64 NHPs	**Timeline (Months)**	**Progress**	**Site 1** Prof. Vijay K. Singh AFRRI	**Site 2** Prof. Martin Hauer-Jensen UAMS
Major Task 3. Radioprotective efficacy of GT3 against two different doses of radiation: ***Whole body exposure***	Months			
Subtask 1. Studies with 5.8 Gy: Irradiation, monitoring of health conditions, CBC and blood biochemistry, gut bacterial translocation, cytokine profile, citrulline analysis, flow cytometric phenotyping of hematopoietic cells, hematopoietic and stem cell studies, gastrointestinal studies, vascular/endothelial studies, histopathology, necropsy	16-22	Complete	Dr. V. K. Singh	Prof. Hauer-Jensen
Subtask 2. Studies with 6.5 Gy: Irradiation, monitoring of health conditions, CBC and blood biochemistry, gut bacterial translocation, cytokine profile, citrulline analysis, flow cytometric phenotyping of hematopoietic cells, hematopoietic and stem cell studies, gastrointestinal studies, vascular/endothelial studies, histopathology, necropsy	23	Ongoing	Dr. V. K. Singh	Prof. Hauer-Jensen
Milestone(s) Achieved: Efficacy of optimal formulation	36		Dr. V. K. Singh	Prof. Hauer-Jensen

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments

Major Task 1 & 2

During this annual reporting period, we completed all remaining analysis for Major Tasks 1 and 2, including thrombomodulin, flow cytometry/CFU assays, and PK analysis. As previously reported, animal work for Major Task 1 and 2 was completed during the last annual reporting period.

Flow cytometric phenotyping of hematopoietic cells

Bone marrow samples were collected under aseptic conditions from the 4 animals used in the PK study. Two ml of bone marrow cells were aspirated from the NHP's iliac crest using a bone marrow aspiration device, and then suspended in RPMI 1640 medium with 20% fetal bovine serum and penicillin-streptomycin in a cell culture tube. The sample was placed in wet ice and shipped immediately to our collaborator for immunophenotyping by flow cytometry as well as Colony-Forming Unit Assays. Flow cytometry data was reported previously in the previous annual report and Major Task 1 and 2 completion report. CFU assays were completed using the methodology described below:

Colony-Forming Unit (CFU) Assays

Colony-forming unit (CFU) assays in bone marrow samples were measured. To prepare the bone marrow nucleated cells (BMCs), red blood cells (RBCs) were lysed in the bone marrow specimens from NHP samples with a RBC lysis solution from BD Biosciences (Cat# 555899) according to the manufacturer's instruction. The resulting BMCs were washed with PBS and re-suspended in DMEM medium with 10% FBS. After counting cell numbers, the cell concentration was adjusted to 2.5×10^5/ml. A volume of 0.2 ml of the cells (5×10^4) were mixed with 2 ml MethoCult™ H4034 Optimum (StemCell Technologies, Vancouver, BC, Canada). The cells were seeded into wells of a 24-well plate in triplicate for each NHP. Colonies of burst-forming unit-erythroid (BFU-E) and CFU-granulocyte macrophage (CFU-GM) were scored on day 14 of the incubation according to the manufacturer's protocol. The raw data is presented in Appendix A, Table 1.

Thrombomodulin (TM) Analysis

Plasma samples taken from the 4 NHPs were analyzed for thrombomodulin. Thrombomodulin is an integral membrane protein that is expressed on the surface of endothelial cells that serves as a cofactor for thrombin. At the time of euthanasia, blood for plasma was collected into EDTA tubes and centrifuged at 1,000 RPM at 4 ºC for 30 min. The supernatant was collected, aliquoted, and stored at -80 ºC until it was shipped to the collaborator for analysis at UAMS.

Two different TM ELISA kits from two different vendors (MyBioSource.com, San Diego, CA and Elabscience, Beijing, China) were tried, and one kit was selected from Elabscience, Beijing, China, Cat. No. E-EL-MK1229). NHP plasma sample dilution (1:100) for TM ELISA analysis was standardized. TM levels in 4 unirradiated plasma samples from the PK-PD study (RA0461F, RA0516F, RA0746F, and RA0861M) were analyzed. In addition, two archival NHP plasma samples were used to serve as the controls: one sample was from one day before 8 Gy partial body irradiation (PBI) and the other sample was from one day after 8 Gy PBI.

Plasma samples were diluted to 1:100 using Reference Standard & Sample Diluent supplied by the manufacturer. The 100 µl standard or samples were incubated at 37 °C onto a microtiter plate coated with monoclonal antibodies which binds the TM for 1.5 h. After incubation, the liquid in each well was removed and Biotinylated Detection Ab working solution specific for TM and Avidin-Horseradish Peroxidase (HRP) conjugate was added to each well successively and incubated. Afterwards, the free components were washed away and the substrate solution was added to each. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution. The color intensity was determined by absorbance at 450 nm using an ELISA plate reader (Bio-TEK, Synergy HT multi-detection microplate reader) and was proportional to the concentration of TM in the standards and samples. The ELISA standard is provided in figure 29. A standard 2-parameter curve fit was used to determine the plasma TM concentrations. TM was duplicate measured for each sample. Data is expressed as group mean ± standard deviation of the mean (STDEV). (Appendix A, Table 2, Figure 1-3)

Plasma samples for PK analysis were collected at AFRRI and analyzed by Craft Technology using the methodology described below.

PK Analysis for GT3

Plasma samples were stored at -80 °C before the analysis. For the determination of γ-tocotrienol, plasma samples were thawed at room temperature, 2.5 µg of δ-tocotrienol (internal standard) in 25 µl methanol, 500 µl of acetonitrile: tetrahydrofuran (3:2, v/v) were added, and the samples were mixed by vortexing for 5 min. The samples were centrifuged (12,800 g for 20 min at 10 °C), and the supernatant was transferred to an amber vial and dried under nitrogen. To improve the recovery of the tocols, the pellet was suspended in 100 µl hexane, vortexed, centrifuged (12,800 g for 10 min at 10 °C) and the supernatant was transferred to the same vial. The dried residues were analytically transferred to deactivated glass micro-inserts using methylene chloride, dried under nitrogen, and derivatized using N-methyl-N-TMS trifluoroacetamide at 25 °C. The derivatized samples were quantitated in triplicate by gas chromatography/mass spectrometry (5975 GC/MSD, Agilent) using single-ion monitoring and a 30-m HP-5MS column (0.250 mm, 0.25 µm).

Samples were analyzed using helium as the carrier gas (head pressure of 27 psi), 1 µl split less injection. The injector temperature was 275 °C, the column temperature was maintained at 220 °C for 2 min followed by a gradient of 25 °C/min to 300 °C, and remained at that temperature for 10 min. The transfer line temperature was maintained at 285 °C for 13.5 min followed by a gradient of 25 °C/min to 300 °C, and remained at that temperature for 10 min. The conditions were: electron impact, source temperature 230 °C, quadrupole temperature 150 °C, and ionization voltage 70 eV."

The data from these analyses are summarized in Appendix A, Figure 4. .

Major Task 3

During this annual reporting period, we have completed four cohorts of Major Task 3 "Radioprotective efficacy of GT3 against two different doses of radiation" The initial stages of Major Task 3 began with the procurement of NHPs and the test article (gamma-tocotrienol (GT3)) for evaluation.

We completed subtask 1 with 5.8 Gy in three cohorts. Cohort I was comprised of 10 NHPs (9 males and 1 female); cohort II was comprised of 12 NHPs (11 males and 1 female) and cohort III had 10 NHPs (10 females). Subtask 2 with 6.5 Gy is in progress with one cohort of 16 NHPs already complete at this time. Details for each cohort of 5.8 Gy including irradiation details, abdominal measurements, weights, and NHP ID numbers are provided in Appendix B, Table 1-3. It is important to note that, Cohort II with 16 NHPs will be initiated sometime in the near future to complete subtask 2. We will provide experimental details of data from the experiment with 6.5 Gy once both cohorts have been completed.

For all cohorts, blood samples were collected for standard analysis including complete blood count (CBC), biochemistry, and cytokine analysis. Samples for these analyses were collected, processed, and analyzed at AFRRI. The blood collection schedule for Major Task 3 has been provided in Appendix B, Table 4.

In addition, we collected blood plasma, bone marrow, and intestinal tissue samples for flow cytometric phenotyping of hematopoietic cells, hematopoietic and stem cell studies, and gastrointestinal studies. These samples were collected at various time points and shipped to our collaborators at UAMS for analysis (Appendix G) Results and data for subtask 1 with 5.8 Gy is summarized in the report below and provided in Appendices C-G.

Survival

In the efficacy study with 5.8 Gy, we observed an 81 % survival rate (13/16) in the GT3-treated NHPs compared to a 63% survival rate (10/16) in vehicle-treated NHPs. The survival figure has been presented in Appendix C, Figure 1.

CBC results

We analyzed 11 blood parameters in the efficacy study with 5.8 Gy: white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), platelets, neutrophils, lymphocytes, monocytes, eosinophils, basophils, and reticulocytes. CBC data from this study is presented in Appendix C, Figures 2 - 3.

WBC counts followed a similar trend across all cohorts during the 60 d study. On SD 4, counts are significantly higher in the vehicle-treated group. For both the GT3 and vehicle-treatment groups, the nadirs fell on SD 14. WBC counts returned to baseline values by SD 24 for both treatment groups.

CBC results (continued)

In regards to RBC, HGB, and HCT levels, both treatment groups follow a similar trend throughout the 60 d study. On SD 18, the GT3-treated group had significantly higher counts than the vehicle-treated group. Both drug and vehicle treatment groups reach their lowest values at SD 20 and returning to their pre-irradiation values by SD 60.

Platelet counts in irradiated animals at other time points followed similar trends between vehicle control and GT3-treated groups with the nadir falling on SD 14. Vehicle-treated animals were severely thrombocytopenic from SD 12-14. Similarly, GT3-treated animals were thrombocytopenic after SD 12 and severely thrombocytopenic on SD 14. Both the vehicle-treated and GT3-treated group recovered from thrombocytopenia by SD 16. Platelet counts in both treatment groups stabilize by SD 26.

Neutrophil counts followed a similar pattern in both the drug and vehicle-treated groups. The lowest neutrophil counts are on SD 14. The vehicle-treated group was neutropenic from SD 6-12 and on SD 16 and they were severely neutropenic on SD 14. Platelet counts were significantly higher in the vehicle-treated group for two time points (SD 4 and 8). Similarly, the GT3-treated group was neutropenic from SD 6-16. At no point during the study was the GT3-treated group considered severely neutropenic. Platelet counts were significantly higher in the GT3-treated group at SD 12. The vehicle-treated and GT3-treated groups both recovered from neutropenia on SD 18.

Lymphocyte counts followed a unique pattern from SD -3, counts decreased substantially, reaching the lowest point by SD 16, for both treatment groups. Values progressively increased after SD 18 but did not meet the baseline values by SD 60. On SD 30, 34, and 38, lymphocyte counts were significantly higher in the GT3-treated group than the vehicle-treated group. These significant differences were not noted at any other time point during the study.

Monocyte counts exhibited a unique trend, counts decreased post-irradiation in both treatment groups, with the nadir falling on SD 12 and 14 for the vehicle-treated and GT3-treated groups, respectively. On SD 2, 4, and 6, the vehicle-treated group reported significantly higher monocyte counts than the drug treated group. Monocyte counts slowly return to their baseline (pre-irradiation) values by SD 20 in both treatment groups. Monocyte counts in the GT3-treatment group supersede their baseline values by SD 60, this was not observed in the vehicle-treated group

Eosinophil counts decreased from SD -3, reaching minimum values at SD 12. Following SD 26, counts increased reaching their apex at SD 28 for vehicle-treated and SD 42 for GT3-treated subjects. Basophil counts followed a unique pattern, in both the vehicle and drug-treated groups, throughout the 60 d study. Counts decreased from SD -3 to SD 8, with a brief increase on SD 4. On SD 6, counts were significantly higher in the vehicle treated group. This difference is not noted at any other time point in the study. After SD 16, counts gradually increased coming to their highest value at SD 24. On SD 10, 18 and 24, basophil counts were significantly higher in the GT3 treatment group. Counts gradually decrease after SD 24 returning to baseline values by SD 60.

Reticulocyte counts decreased to their lowest point by SD 6 but surpassed baseline values by SD 26. On SD 1, 12 and 14, the GT3-treated group had significantly higher reticulocyte counts than the control group. On SD 4 and 6, the vehicle-treated group reported significantly higher reticulocyte counts than the GT3-treated group. After SD 26, counts progressively decreased, returning to baseline values by SD 60

Biochemistry results

We analyzed 16 different blood chemistry parameters throughout the course of the efficacy study with the 5.8 Gy dose: glucose, albumin, ALT, AST, ALKP, total bilirubin, total protein, GGT, creatinine, uric acid, sodium, potassium, chloride, calcium, LDH, and triglycerides (Appendix D, Figures 1-3).

In respect to blood glucose concentration, levels remained consistent from their pre-irradiation values throughout the study with the exception of a slight decrease seen on SD 28 for both treatment groups. On SD 50, a decrease was observed in the vehicle-treated group, this difference was statistically significant. Similarly, albumin concentration remained consistent between treatment groups, though on SD 2, the GT3-treated group had significantly lower albumin concentration than the control group. ALT concentrations peaked on SD 2 and then gradually decreased until SD 28, from there they steadily increased until SD 60 where they superseded pre-irradiation levels, in both treatment groups. AST concentrations peaked on SD 2 and SD 50 for both groups, rapidly decreasing and returning to baseline values. On SD 2, the GT3-treated group reported significantly higher AST concentrations than the vehicle-treated group.

ALKP levels remained consistent from SD -3 to SD 28. On SD 28, the GT3-treated NHP ALKP levels superseded the vehicle-treated NHP levels until SD 60. Total bilirubin levels followed an upwards trend from SD -3 to SD 2, but decreased sharply from SD 2 to SD 28. After SD 28, both treatment groups increased to near pre-irradiation levels. On SD 60, the GT3-treated group reported significantly higher total bilirubin concentration when compared to the vehicle-treated group. In regard to blood total protein concentrations, levels decreased from SD -3 to SD 2, and more sharply in the GT3-treated group. This difference was statistically significant. Levels then increased gradually until SD 60 though not superseding pretreatment values.

Blood GGT concentration gradually decreased for both treatment groups until SD 28, then increased to near pre-irradiation values. On SD 2, the vehicle-treated group had significantly higher GGT levels than the drug-treated group. Blood creatinine levels followed an almost perfect linear trend throughout the 60 d study with the exception of a peak observed in the GT3-treated group on SD 2.

Blood uric acid concentration also followed an almost consistent linear trend for the duration with the exception of a minor increase in the vehicle-treated group on SD 38. In respect to blood sodium concentration, maximum values were observed on SD -3, these levels decreased sharply on SD 2, increased from SD 2 until SD 38, and finally decreased for the remainder of the study. Concentrations were well below pre-treatment values on SD 60.

Potassium remained consistent and largely linear for both groups with the exception of SD 2 and SD 38 where the vehicle-treated group levels were greater. Likewise, blood chloride concentrations declined from SD -3 to SD 38 then in GT3-treatment groups while initially increasing from SD -3 until SD 2 before declining in the vehicle-treatment group. Both treatment groups increased gradually to near pre-treatment values by SD 60. LDH levels were consistent throughout the study with the exception of a sharp peak seen in the GT3-treated group on SD 2; this difference was statistically significant. Triglycerides peaked in the GT3 treatment group on SD 2; this difference was statistically significant. The vehicle-treated group triglyceride levels remained fairly consistent, though it gradually increased over time.

Cytokine results

We analyzed a total of 44 cytokines throughout the course of the efficacy study with the 5.8 Gy dose: IL-1ß, IL-1ra, IL-2, IL-4 ,IL-5, IL-6 , IL-7, IL-8, IL-9 , IL-10, IL-12(p70), IL-13, IL-15, IL-17, Eotaxin, FGF basic, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1(MCAF), MIP-1α, PDGF-bb, MIP-1ß, RANTES,B TNF-α , VEGF, IL-1α, L-2Rα, IL-3, IL-12p40, IL-16, IL-18, CTACK, GROa, LIF, M-CSF, MIF, MIG, SCF, SCGF-, SDF-1α, TNF-ß, and TRAIL. Overall, it was observed that GT3 administration did not induce high levels of cytokines in NHPs we used in the subtask 1 study (Appendix E. Figures 1-7).

However, noted spikes were observed in a few cytokines at various time points. At 4 h post-administration, GT3 induced high levels of cytokines in IL-6 and G-CSF. On SD 1, GT3 induced high levels of GM-CSF. Similarly, at SD 1 and 3, there was a noted spike in IL-18 levels, in NHP treated with GT3.

Vital Signs Results

In addition to collection blood samples, vital signs were taken at each time point to monitor the overall health condition of the animals. The following vital signs were recorded: pulse, blood pressure, weight, and temperature. Temperature was taken using a rectal probe on SD -7, -3, and -1; for the remainder of the time points was taken using the DAS-7006/7r scanner with implanted chip. Heart rate and blood pressure were recorded using a Surgivet Advisor vital sign monitor. Weight was recorded using an Ohaus digital platform scale. No substantial or consistent changes in vital signs parameters were observed in either treatment group (Appendix F, Figure 1). It is important to note that the highest temperature was observed at the pre-irradiation time points. This is likely due to the stress response associated with the pole and collar restraint method used in NHP handling.

Sample collection for various analyses

Following the completion of each cohort, animals were euthanized 60 d post-irradiation. Bone marrow and jejunum tissue samples were collected and sent to our collaborator at UAMS. Samples collected for the analysis of for flow cytometric phenotyping of bone marrow cells, TM/ D-Dimer, citrulline, calprotectin, and for intestinal histology (Appendix G and Figures 1-6).

Flow cytometric phenotyping of hematopoietic cells

Bone marrow samples were collected under aseptic conditions from all the animals used in the efficacy study. Two ml of bone marrow cells were aspirated from the NHP's iliac crest using a bone marrow aspiration device and then suspended in RPMI 1640 medium with 20% fetal bovine serum and penicillin-streptomycin in a cell culture tube. The sample was placed in wet ice and shipped immediately to our collaborator for immunophenotyping by flow cytometry.

Once these samples arrived to the collaborator, red blood cells were lysed with lysis buffer. The number of bone marrow nucleated cells (BMCs) were counted using a cell counter (Heska, Loveland, Colorado). BMCs were labeled with anti-CD45-FITC (eBioscience, San Diego, CA) and anti-CD34-PE (BD) antibodies after incubation with anti-CD16/32 to block the Fcγ receptors (eBioscience). After washing, the cells were resuspended in phosphate buffer saline (PBS) containing 0.25 μg/mL propidium iodide (PI, Sigma, St. Louis, MO) to evaluate the viability of the cells and to exclude dead cells from $CD45^+$ and/$CD34^+$ cell analysis using a LSRII flow cytometer (Becton Dickinson, San Jose, CA). A fraction of BMCs from each NHP was frozen in a liquid nitrogen tank after the cells were suspended in a cell frozen medium (DMEM medium supplemented with 10% FBS and 10% DMSO) for future use. Results from these analyses are presented and summarized in Appendix G, Figure 1.

CFU assays in bone marrow samples were also measured using the same methodology as described previously in Major Task 1 and 2. The results for this assay are presented in Appendix G, Table 1.

D-Dimer Analysis

As part of our hematopoietic studies using GT3, plasma samples were collected at various time points (SD -1, 3, 7, 14, 22, 30, and 60) were analyzed for D-Dimer. D dimer is a fibrin degradation product, a small protein fragment present in the blood after a blood clot is degraded by fibrinolysis. Blood samples were collected into tubes containing 3.2% sodium citrate and kept on wet ice pending centrifugation. Within 30 min of collection, blood specimens were then centrifuged under refrigeration (2-8^{0}C at 3000 g) for 10 min. The supernatant was collected, aliquoted, and stored at -80 ºC until it was shipped to the collaborator for analysis at UAMS.

Results from the analysis are summarized in (Appendix G, Figure 2). Results indicate that there was little to no difference between GT3-and vehicle treated NHPs in D-Dimer levels.

TM Analysis

Plasma samples were also collected at various time points (SD -1, 3, 7, 14, 22, 30, and 60) for Thrombomodulin analysis. Thrombomodulin is an integral membrane protein that is expressed on the surface of endothelial cells that serves as a cofactor for thrombin. . Blood samples were collected into tubes containing 3.2% sodium citrate and kept on wet ice pending centrifugation. Within 30 min of collection, blood specimens were then centrifuged under refrigeration (2-8^{0}C at 3000 g) for 10 min. The supernatant was collected, aliquoted, and stored at -80 ºC until it was shipped to the collaborator for analysis at UAMS.

Results from the analysis are summarized in (Appendix G, Figure 3). Results indicate that there was little to no difference between GT3-and-vehicle treated NHPs in TM levels.

Calprotectin Analysis

Fecal samples were collected at various time points (SD -3, -1, 3, 5, 14, 22, 30, and 60) for calprotectin analysis. Calprotectin analysis indicates intestinal inflammation. Elevated calprotectin levels are indicative of intestinal inflammation and the degree of elevation is associated with the severity of the inflammation.

Results from the analysis are summarized in (Appendix G, Figure 4). Results indicate that there was little to no difference between GT3-and vehicle treated NHPs in calprotectin levels.

Citrulline Analysis

Plasma samples were also collected at various time points (SD -1, 3, 7, 14, 22, 30, and 60) for citrulline analysis.
Plasma citrulline levels are an indicator of intestinal mucosal integrity and subsequent radiation-induced damage. Plasma citrulline is also considered a good biomarker for functional enterocyte mass.

Results from the analysis are summarized in (Appendix G, Figure 5). Results indicate that there was little to no difference between GT3-and vehicle treated NHPs in citrulline levels.

Micronuclei Analysis

1.0 mL of whole blood was collected in sodium heparin tubes and shipped to our collaborators at Columbia University for micronuclei analysis. Micronuclei (MNi) are small, round objects found in the cytoplasm of cells outside the main nucleus and represent chromosome fragments or whole chromosomes that are not incorporated into the daughter cell after nuclear division. Analysis is limited to binucleated cells (BN) in order to allow for the selection of lymphoycytes that have divided once.

The CBMN assay used to measure the MNi per BN, measures chromosome breakage, DNA misrepair, chromosome loss, non-disjunction, necrosis, apoptosis, and cyostasis.
Micronuclei analysis results did not indicate a significant difference in micronuclei per BN in GT3-treated NHPs versus vehicle-treated NHPs. Results are summarized in Appendix G, Figure 6

Intestinal histology study

Study staff received advanced training at CiTox laboratories in Ontario, Canada for intestinal tissue collection. Study staff were trained on full tissue collection, crypt/mucosal intestine collection, frozen sections, RNA later and bone marrow smears. Upon return their return to AFRRI, study staff were able to successfully harvest jejunum sections from surviving animals (SD 60) in the efficacy study with 5.8 Gy, for future analysis for histology. Jejunum was collected using the technique described above and as detailed in Amendment 7. Amendment 7 was recently approved for the collection of GI tissue as a surgical procedure under deep anesthesia before euthanasia, in study NHPs. This is a terminal procedure, and the animals are euthanized immediately following GI tissue collection

Collection procedure details:

Animals were fasted for 8-12 h before sedation. Water was not restricted, and animals had free access to water at all times. The NHPs were chemically sedated with ketamine (5-15 mg/kg im) with (needle 22-25 G). Animals were then inducted, 3-5% isoflurane in 100% oxygen by mask. For maintenance, 1-3% isoflurane was administered in 100% oxygen via endotracheal tube. While the animal was under the anesthesia, vital signs were monitored including SpO2, pulse, respiration rate and body temperature. A Bair Hugger heated surgical table was used for keeping animals warm during the procedure. The animals body temperature were recorded frequently using a rectal thermometer or via microchip throughout the procedure.

Collection procedure details continued:

Animals were then sedated, maintained anesthetized, and monitored continuously. The animal's chest and abdomen was shaved and wiped clean with 70% alcohol. The animal was moved to the surgical suite. An incision, large enough to access the abdominal cavity, was made below the animal's diaphragm. Sections of intestine were be isolated (clamped at two ends) and excised for further processing. Excessive bleeding was mitigated via cautery pen. Immediately following removal of the GI tissue, animals were euthanized after tissue collection and full necropsies were performed to collect remaining tissue samples (heart, lung, sternum, spleen, liver, kidney, jejunum, duodenum, ileum, large intestine, and bladder).

At this time, all intestinal tissues and samples have been collected and once they have been processed and they will be analyzed by board certified histopathologist, we will be able to report these results in our final report for this grant.

Summary

In summary, at this stage of the project, all animal work for subtask 1 using 32 NHPs at a dose of 5.8 Gy has been completed. Work for subtask 2 is ongoing, with cohort I with 16 NHPs already complete. We will continue moving forward with the project and expect to complete subtask 2 of this study in the very near future.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Research assistants working in the PI's laboratory had a unique opportunity work with the NHP model. This is rare opportunity as only limited laboratories in US carry out research using the NHP model. Two research assistants previously working in small animal hospitals joined PI laboratory to pursue this opportunity. During the summer, we had the opportunity to host five students (both high school and college) to work as interns in the PI's laboratory and to receive valuable educational training. We had one intern each from the SEAP, NREIP, MIDN, HJF and Infused Solutions intern programs. They had ample opportunities to participate in various assays and data analysis. This project is going to aid several junior researchers in their career advancement. All staff members working in the PI's laboratory are receiving training for various assays used in this project. PI staff members take care of NHPs including but not limited to daily dietary enrichment, monthly occupational/sensory enrichment and critical period monitoring and observations. Additional staff will be recruited for this project in due course of time.

In addition, three research staff members in the PI's lab had the opportunity to attend training at CiTox laboratories in Ontario, Canada for necropsy collection training. PI staff were trained in full tissue collection, crypt/mucosal intestine collection, frozen sections, RNA later and bone marrow smears. At the end of our PK study, we encountered some issues with tissues collection and discussed these issues extensively with our collaborators. It was then decided that our staff should attend training to improve the quality of our sample collection.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Research with GT3 was shared with scientific community in a recently published review article in the International Journal of Molecular Sciences. The PI delivered a talk during Military Health Science Research Symposium at Orlando in August 2016 where GT3 radioprotective efficacy was discussed. GT3 is the only radiation countermeasure (specifically radioprotector – administered before radiation exposure) under development for which US DoD has intellectual property rights.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

The PI will try to publish research findings in a peer review journal. Usually, it takes longer time to generate data for a good publication using NHP model. The PI will also attend meetings to present project findings.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to Report.

It is too early to discuss such impacts. This project has only completed two years of its scheduled four years. Being a NHP model study, progress is gradual. We will have significant findings during next year and such findings will have impact in scientific community. Only limited numbers of radiation countermeasures have reached to NHP model for evaluation and GT3 is one of those agents.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."
Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

No additional changes in approach have been made and we do not anticipate any changes in the near future.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

A new IACUC protocol was written and submitted to complete Specific Aim 3 (Major tasks 4 and 5 of SOW, and experiment 3 of the ongoing approved IACUC protocol # P-2015-01-001). Specific Aim 3 experiments of this project was part of our ongoing protocol P-2015-01-001. The ongoing protocol will expire before the Specific Aim 3 experiments could be initiated. Though this research project is for 4 years, associated approved IACUC protocol P-2015-01-001 is for three years. In addition, IACUC protocol was approved 6 months ahead of project funding which gives only two and half years' time to do experiments. IACUC protocol is always approved for a three year period. Further, IACUC protocol cannot be extended under any situation. Thus, any IACUC protocol period of operation is always for three years. Hence, additional time is required to complete the remaining experiments of this research project.

In brief, two consecutive IACUC protocols will be needed to complete animal work of this project. The study proposed in the new protocol was previously approved, no new animal studies are being proposed in new protocol. Animals used for experiment # 3 of ongoing IACUC protocol P-2015-01-001 will not be used in that protocol. Those animals will be used in the new protocol which has been submitted and has received approval by AFRRI IACUC and has gone to ACURO for 2nd tier approval

A copy of this protocol for experiment 3 was provided and submitted earlier as a supplementary file with the month 24 monthly report.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

There has been no significant impact on expenditures.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

There are no human subjects used at any point in this study.

Significant changes in use or care of vertebrate animals.

There has been no significant change in the care or use of vertebrate animals. The exact number of animals for use in this study has changed without any significant impact on the study.

Significant changes in use of biohazards and/or select agents

There has been no significant change in the care or use of biohazard or select agents.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no*

Publication of several review articles:

1) Singh VK, Seed TM. A review of radiation countermeasures focusing on injury-specific medicinals and regulatory approval status: part I. Radiation sub-syndromes, animal models and FDA-approved countermeasures. Int J Radiat Biol. 2017 Jun 26:1-19. doi: 10.1080/09553002.2017.1332438. [Epub ahead of print] PubMed PMID: 28650707.
   i) Acknowledgement of federal support: Yes
2) Singh VK, Garcia M, Seed TM. A review of radiation countermeasures focusing on injury-specific medicinals and regulatory approval status: part II. Countermeasures for limited indications, internalized radionuclides, emesis, late effects, and agents demonstrating efficacy in large animals with or without FDA IND status. Int J Radiat Biol. 2017 Jun 28:1-15. doi: 10.1080/09553002.2017.1338782. [Epub ahead of print] PubMed PMID: 28657406.
   i) Acknowledgement of federal support: Yes
3) Singh VK, Hanlon BK, Santiago PT, Seed TM. A review of radiation countermeasures focusing on injury-specific medicinals and regulatory approval status: part III. Countermeasures under early stages of development along with 'standard of care' medicinal and procedures not requiring regulatory approval for use. Int J Radiat Biol. 2017 Jun 28:1-22. doi: 10.1080/09553002.2017.1332440. [Epub ahead of print] PubMed PMID: 28657400.
   i) Acknowledgement of federal support: Yes
4) Singh VK, Pollard HB. Ionizing radiation-induced altered microRNA expression as biomarkers for assessing acute radiation injury. Expert Rev Mol Diagn. 2017 Oct;17(10):871-874. doi: 10.1080/14737159.2017.1366316. Epub 2017 Aug 14. PubMed PMID: 28792262.
   i) Acknowledgement of federal support: Yes
5) Singh VK, Olabisi AO. Nonhuman primates as models for the discovery and development of radiation countermeasures. Expert Opin Drug Discov. 2017 Jul;12(7):695-709. doi: 10.1080/17460441.2017.1323863. Epub 2017 May 5. Review. PubMed PMID: 28441902.
   i) Acknowledgement of federal support: Yes

Publications continued:

7) Singh VK, Garcia M, Wise SY, Seed TM. Medical countermeasures for unwanted CBRN exposures: Part I chemical and biological threats with review of recent countermeasure patents. Expert Opin Ther Pat. 2016 Dec;26(12):1431-1447. Epub 2016 Sep 14. Review. PubMed PMID: 27599259.

i) Acknowledgement of federal support: Yes

8) Singh VK, Romaine PL, Newman VL, Seed TM. Medical countermeasures for unwanted CBRN exposures: part II radiological and nuclear threats with review of recent countermeasure patents. Expert Opin Ther Pat. 2016 Dec;26(12):1399-1408. Epub 2016 Sep 9. Review. PubMed PMID: 27610458; PubMed Central PMCID: PMC5152556.

ii) Acknowledgement of federal support: Yes

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: *5*
Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award).*

Name: Prof. Vijay K. Singh
Project Role: Principal Investigator
Nearest person month worked: 4
Contribution to Project: Study Director and has overall responsibility for technical conduct of the study
Funding Support: Federal Employee

Name: Paola Santiago
Project Role: Research Assistant
Nearest month person worked: 12
Contribution to Project: Responsible for the collection and organization of data, performing biochemical assays from NHPs samples and data analysis and interpretation
Funding Support: CDMRP contract
Following staff members were not employed in this project but provided support during accomplishment of this project as and when needed.

Name: Stephen Wise
Project Role: Research Associate
Nearest month person worked: N/A
Contribution to Project: Responsible for the collection and organization of data, performing biochemical assays from NHPs samples and data analysis and interpretation
Funding Support: DMRDP Grant

Name: Oluseyi Fatanmi
Project Role: Research Biologist
Nearest month person worked: N/A
Contribution to Project: Responsible for the collection and organization of data, performing biochemical assays from NHPs samples and data analysis and interpretation
Funding Support: Federal Employee

Name: Melissa Garcia
Project Role: Research Assistant
Nearest month person worked: N/A
Contribution to Project: Responsible for the collection and organization of data, performing biochemical assays from NHPs samples and data analysis and interpretation
Funding Support: DMRDP Grant

Name: Eric Lee
Project Role: Research Assistant
Nearest month person worked: N/A
Contribution to Project: Responsible for the collection and organization of data, performing biochemical assays from NHPs samples and data analysis and interpretation
Funding Support: BARDA Grant

Name: Anne Semon
Project Role: Research Assistant
Nearest month person worked: N/A
Contribution to Project: Responsible for the collection and organization of data, performing biochemical assays from NHPs samples and data analysis and interpretation
Funding Support: BARDA Grant

Name: Amit Verma
Project Role: Post Doctorate Fellow
Nearest month person worked: N/A
Contribution to Project: Responsible for the collection and organization of data, performing biochemical assays from NHPs samples and data analysis and interpretation
Funding Support: NRC

Name: Briana Hanlon
Project Role: Research Assistant
Nearest person month worked: N/A
Contribution to Project: Responsible for the collection and organization of data, performing biochemical assays from NHPs samples and data analysis and interpretation
Funding Support: NIAID Grant

Name: Madison Simas
Project Role: Research Assistant
Nearest person month worked: N/A
Contribution to Project: Responsible for the collection and organization of data, performing biochemical assays from NHPs samples and data analysis and interpretation
Funding Support: NIAID Grant

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:

Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

University of Arkansas for Medical Sciences, Little Rock, Arkansas
Name: Prof. Martin Hauer-Jensen, MD, Ph.D., FACS
Project Role: Collaborator
Researcher Identifier:
Nearest person month worked: 8
Contribution to Project: Dr. Hauer-Jensen is a collaborator for this project, he is working with tissues shared between AFRRI and UAMS. He is responsible for performing analysis for flow cytometric phenotyping of hematopoietic cells, hematopoietic and stem cell studies, gastrointestinal studies, vascular/endothelial studies.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

N/A

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

A quad chart has been enclosed.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Figures and tables mentioned in the report are enclosed as appendices. There are five appendices (A-G) total enclosed in this report.

As stated above, there are eight recently published review articles. They have been included as supplementary files.

Appendix A: Major Task 1 &2 Analysis

Table 1. CFU Assays from PK NHPs

NHP Number	No. of BMCs/well	No. of CFU-GM			No. of BFU-E			No. of CFU-GEMM		
RA0861M	5*10E4	13	11	11	2	0	0	0	0	0
RA0516F	5*10E4	67	41	80	5	8	6	5	6	6
RA0461F	5*10E4	58	68	81	5	5	3	3	3	4
RA0746F	5*10E4	36	39	43	7	6	4	4	3	4

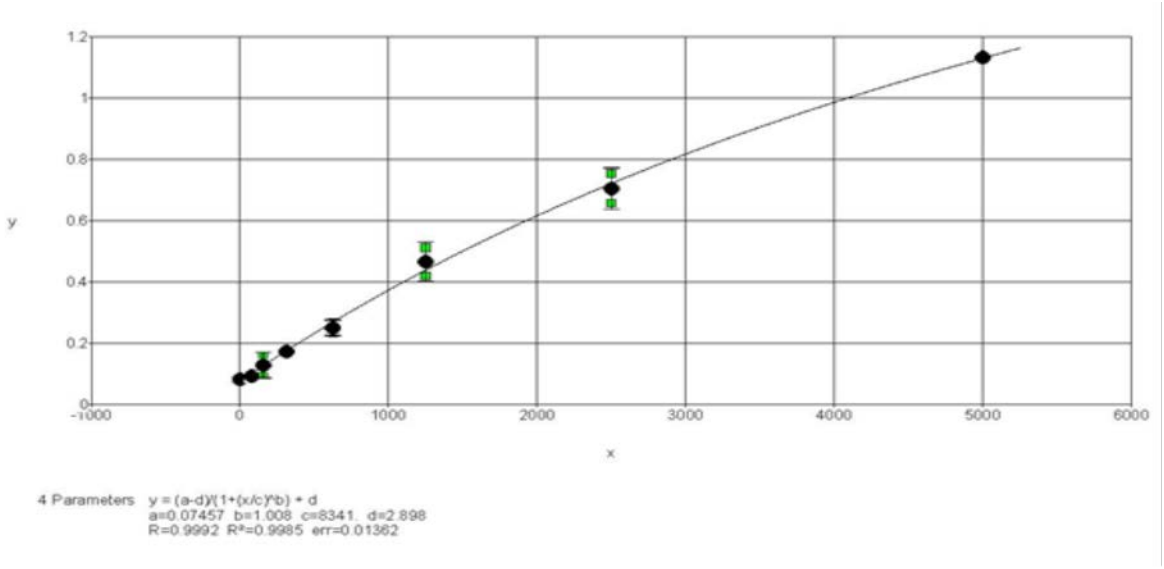


Figure 1: **Thrombomodulin ELISA standard.**

Table 2. Raw data for plasma thrombomoduin (TM) levels in PK Animals.

Animal no.	TM level (ng/ml)
RA0461F	5.1713
RA0516F	7.68
RA0746F	5.297
RA0861M	6.0683

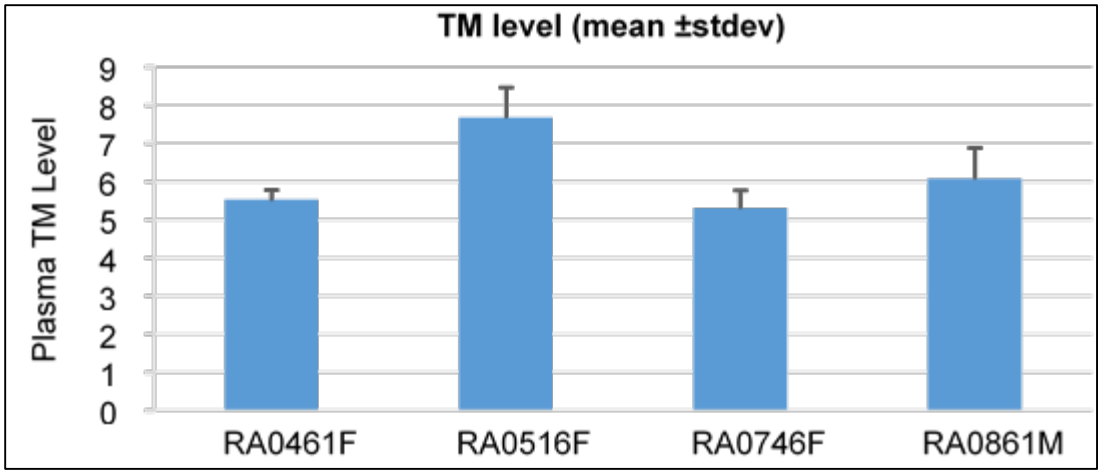


Figure 2: **Plasma TM levels from PK animals**. TM analysis was performed on 4 plasma samples from NHPs administered GT3 (RA0861M, RA0461F, RA0516F, and RA0746).

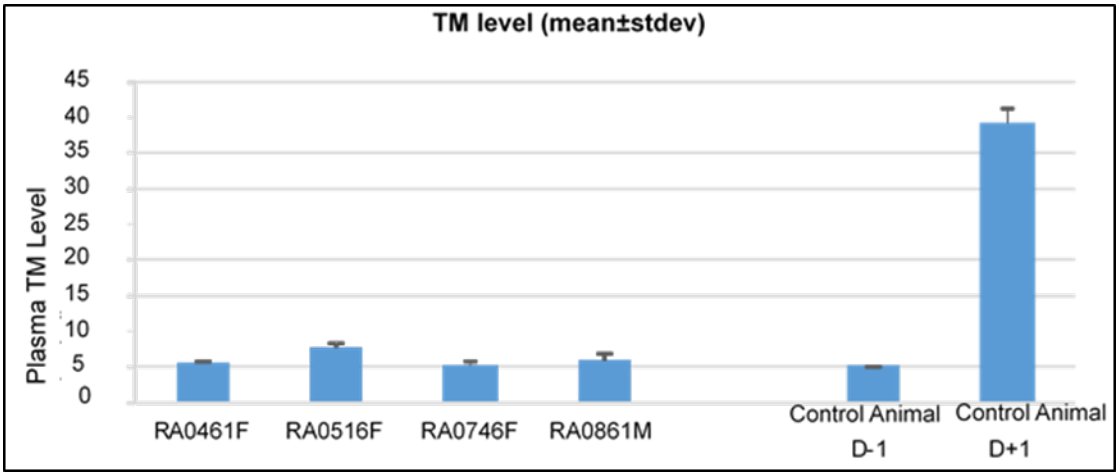


Figure 3: **Plasma Thrombomodulin levels from PK animals vs. control samples** (pre/post exposure to 8 Gy PBI).

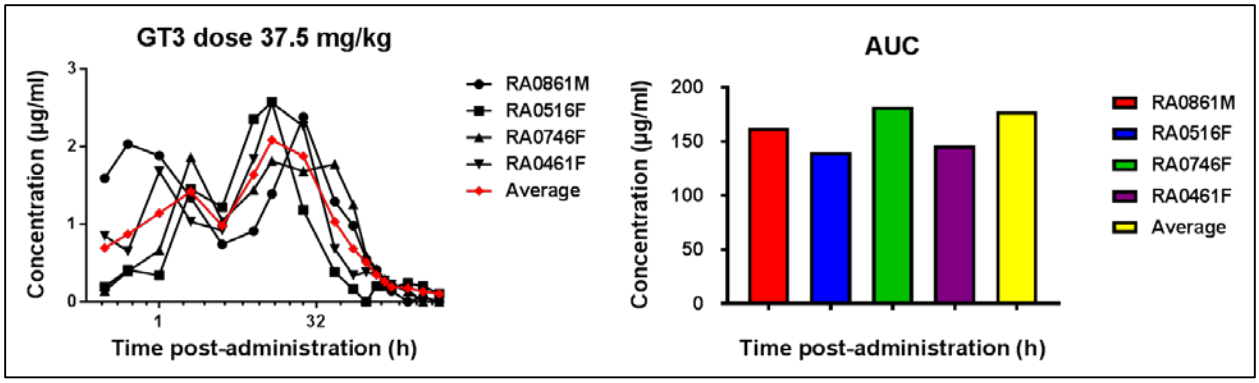


Figure 4: **GT3 pharmokinetic (PK) analysis.** GT3 (37.5 mg/kg) was injected (sc) to 4 NHPs. Samples were collected at various time points following GT3 administration. 150 µL of NHP plasma was provided and analyzed by Craft Technologies in Wilson, NC for pharmokinetics.

Appendix B: Major Task 3 NHP Details

Table 1. NHP radiation and Drug Administration Details-Cohort 1, 5.8 Gy

IACUC Protocol #	2015-01-001
Irradiation Date	11/16/2016

NHP #	Weight (kg)	Lateral measurement (cm)	Radiation Start time	Radiation Stop time	Treatment	Volume Needed (ml), (weight x dose) /concentration (ml)	Volume Drawn/ Injected(ml)	Time Administered (AM/PM)*
RA1413M	6.4	9.4	8:36 AM	8:48 AM	Veh	4.80	4.8	
RA1272M	6.5	9.4			Veh	4.88	4.9	
RA1352M	6.65	9.3	9:07 AM	9:19 AM	Veh	4.99	5.0	
RQ9719M	7.5	9.5			Veh	5.63	5.6	
RA0652M	7.5	10.5	9:37 AM	9:49 AM	GT3	5.63	5.6	
RA1307M	8.1	10.8			GT3	6.08	6.1	
RA0946M	8.95	11	10:05 AM	10:17 AM	Veh	6.71	6.7	
RA1917M	8.15	11			GT3	6.11	6.1	
RA0951M	7.15	10	10:32 AM	10:43 AM	GT3	5.36	5.4	
RA0520F	6.45	9.3			GT3	4.84	4.8	

*Time of administration of drug/vehicle was not recorded for this cohort.

Table 2. NHP radiation and Drug Administration Details-Cohort 2, 5.8 Gy

IACUC Protocol #	2015-01-001
Irradiation Date	11/30/2016

NHP #	Weight (kg)	Lateral measurement (cm)	Radiation Start time	Radiation Stop time	Treatment	Volume Needed (ml), (weight x dose) /concentration (ml)	Volume Drawn/ Injected(ml)	Time Administered (AM/PM)*
RA1360M	4.75	7.9	8:27 AM	8:39 AM	Veh	6.23	6.2	
RA1773M	6.35	8.7			Veh	4.76	4.8	
RA1985M	6.4	9	8:52 AM	9:04 AM	GT3	4.80	4.8	
RA0729F	5.95	9.1			Veh	4.46	4.5	
RA0283M	7.3	9.2	9:21 AM	9:33 AM	GT3	5.48	5.5	
RA1164M	7.25	9.5			Veh	5.44	5.4	
RA1558M	7.35	9.6	9:44 AM	9:56 AM	GT3	5.51	5.5	
RQ9577M	7.75	9.9			GT3	5.81	5.8	
RA1291M	8.15	10.2	10:12 AM	10:24 AM	GT3	6.11	6.1	
RA1330M	7.8	10.2			GT3	5.85	5.9	
RA1889M	8.3	10.3	10:36 AM	10:48 AM	Veh	6.23	6.2	
RA1763M	8.3	10.6			Veh	6.23	6.2	

*Time of administration of drug/vehicle was not recorded for this cohort.

Table 3. NHP radiation and Drug Administration Details-Cohort 3, 5.8 Gy

IACUC Protocol #	2015-01-001
Irradiation Date	3/15/2017

NHP #	Weight (kg)	Lateral measurement (cm)	Radiation Start time	Radiation Stop time	Treatment	Volume Needed (ml), (weight x dose) /concentration (ml)	Volume Drawn/ Injected(ml)	Time Administered (AM/PM)
RA1041F	4.85	7.9	9:00 AM	9:12 AM	Veh	3.638	3.6	9:03 AM
RA0762F	5.55	8.1			Veh	4.163	4.2	9:09 AM
RA2247F	5.1	8.3	9:30 AM	9:43 AM	Veh	3.825	3.8	9:15 AM
RA1671F	5.75	8.6			Veh	4.313	4.3	9:20 AM
RA0714F	5.25	8.4	9:55 AM	10:08 AM	GT3-A	3.938	3.9	9:27 AM
RA0325F	5.6	8.5			GT3-A	4.200	4.2	9:35 AM
RA0705F	5.45	8.8	10:21 AM	10:33 AM	GT3-A	4.088	4.1	10:10 AM
RA2264F	5.35	9.2			GT3-E	4.013	4.0	10:18 AM
RA1809F	6.6	10	10:59 AM	11:11 AM	GT3-E	4.313	4.3	10:26 AM
RA2248F	6.6	9.8			Veh	4.950	5.0	10:35 AM

Table 4. Blood Collection Schedule of Efficacy Study (5.8 Gy)

GT3 dose 37.5 mg/kg	Time of blood draw	CBC 0.5 ml	Cytokine/Biomarkers 2.0 ml	Biochemistry 0.5 ml	Bacteremia 1.0 ml	Citrulline 0.5 ml	Total blood, ml
	-7d	+	+				2.5
	-3d	+	+	+			3.0
	-1d		+			+	2.5
Radiation Dose 5.8 or 6.5 Gy	Day 0, 4 h		+				2.0
	Day 1	+	+				2.5
	Day 2	+	+	+			3.0
	Day 3		+			+	2.5
	Day 4	+					0.5
	Day 5				+		1.0
	Day 6	+	+				2.5
	Day 7		+			+	2.5
	Day 8	+			+		1.5
	Day 10	+					0.5
	Day 12	+	+				2.5
	Day 14	+	+			+	3.0
	Day 16	+					0.5
	Day 18	+					0.5
	Day 20	+					0.5
	Day 22	+	+			+	3.0
	Day 24	+			+		1.5
	Day 26	+					0.5
	Day 28	+	+	+			3.0
	Day 30	+	+			+	3.0
	Day 34	+			+		1.5
	Day 35		+				2.0
	Day 38	+		+			1.0
	Day 42	+			+		1.5
	Day 50	+		+			1.0
	Day 58		+				2.0
	Day 60	+	+	+			3.0

Appendix C: Survival and CBC Results (5.8 Gy)

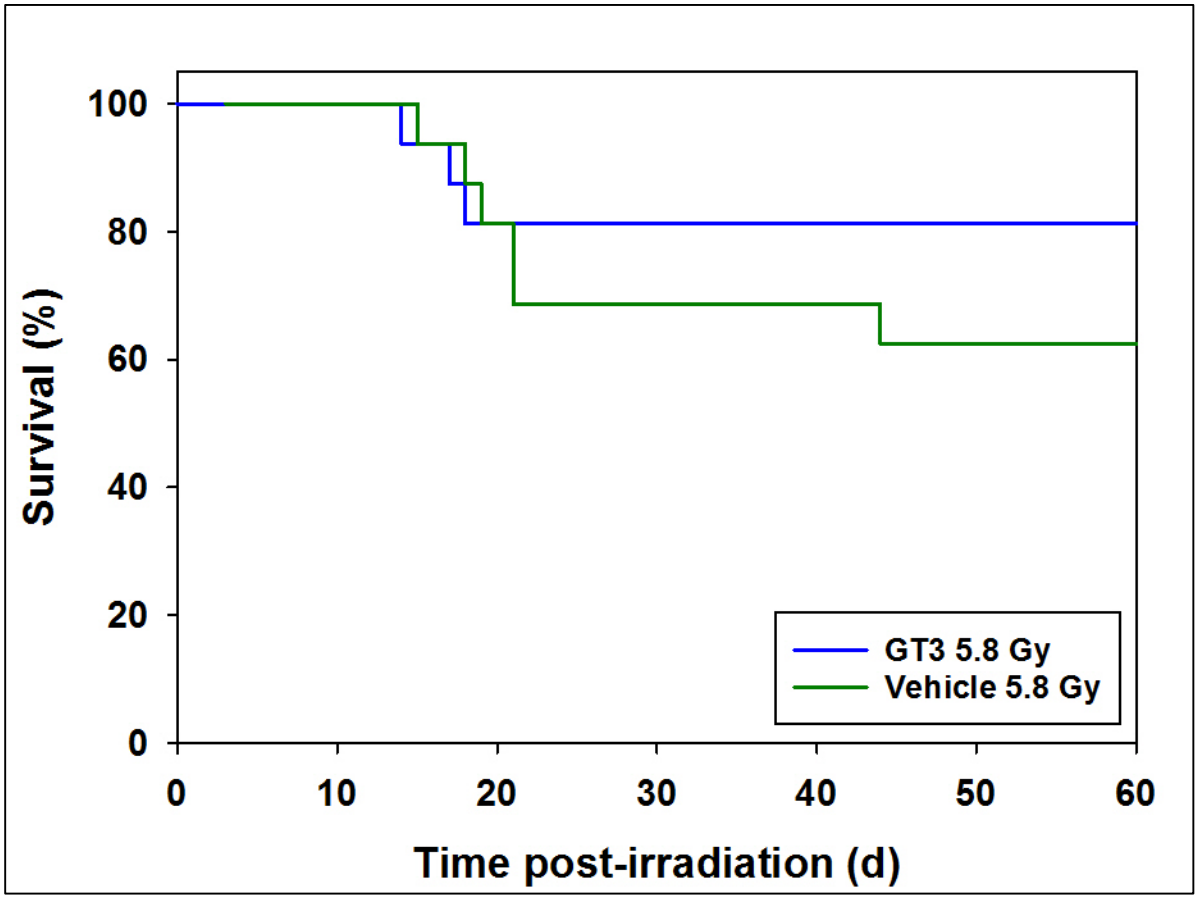


Figure 1: **Survival curve of irradiated NHPs**. Sixteen NHPs were injected with GT3 (37.5 mg/kg) and an additional 16 NHPs were injected vehicle 24 h after exposure to 5.8 Gy (0.6 Gy/min) ^{60}Co γ-radiation.

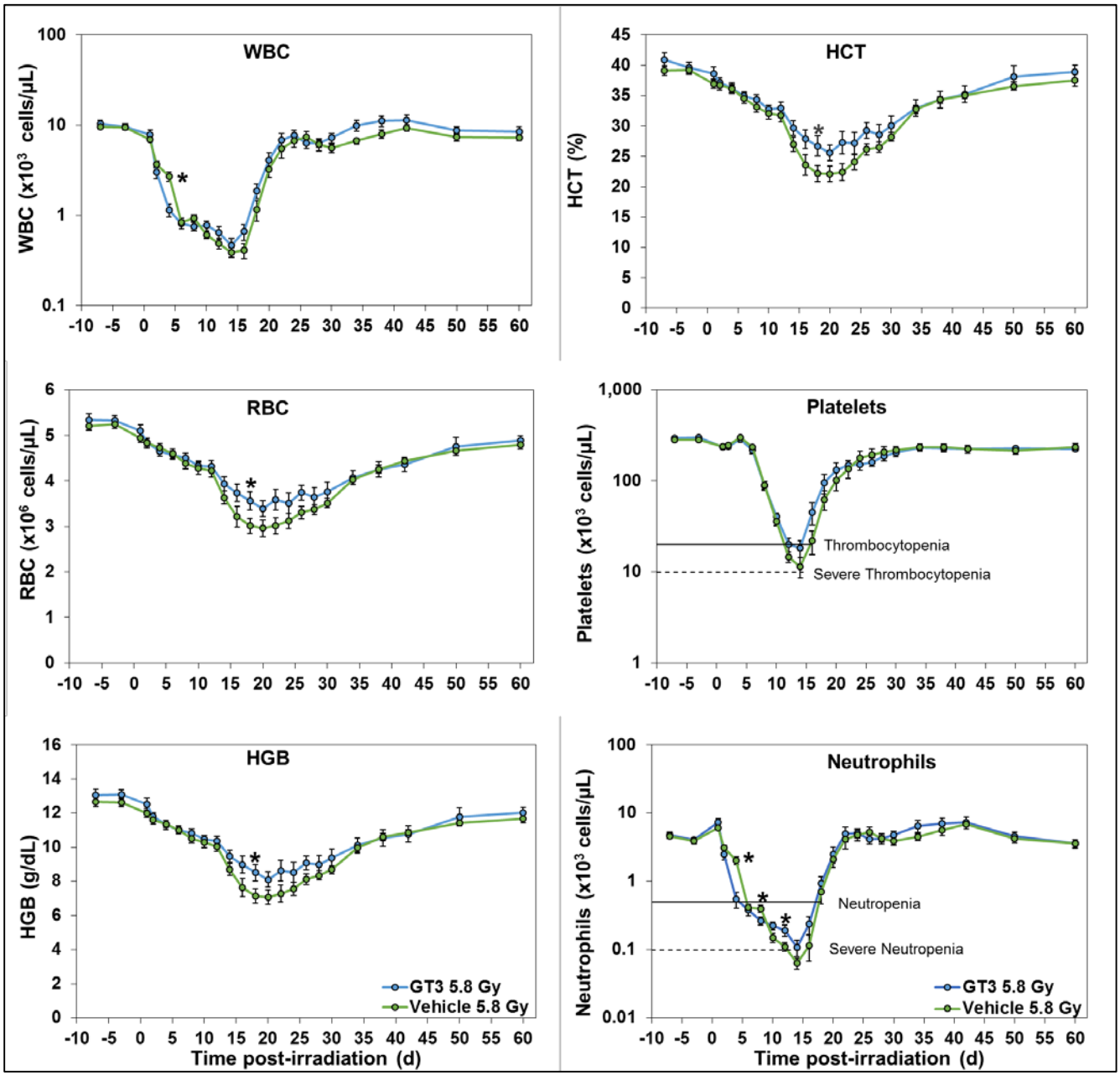


Figure 2: **GT3 induced changes in NHP white blood cells, red blood cells, hemoglobin, hematocrit, platelets, and neutrophils.** Sixteen NHPs were injected with GT3 (37.5 mg/kg) and an additional 16 NHPs were injected with vehicle 24 h before exposure to 5.8 Gy (0.6 Gy/min) ^{60}Co γ-radiation. Blood was collected at various time points and cells were counted using a Bayer Advia-120 cell counter. The data for each time point is presented as the mean ± standard error for each treatment group. The data for each time point is presented as the mean ± standard error for each treatment group. '*' indicates a significant difference between GT3-treated group and vehicle-treated group, when equal variance between groups was assumed. ($p \leq 0.05$).

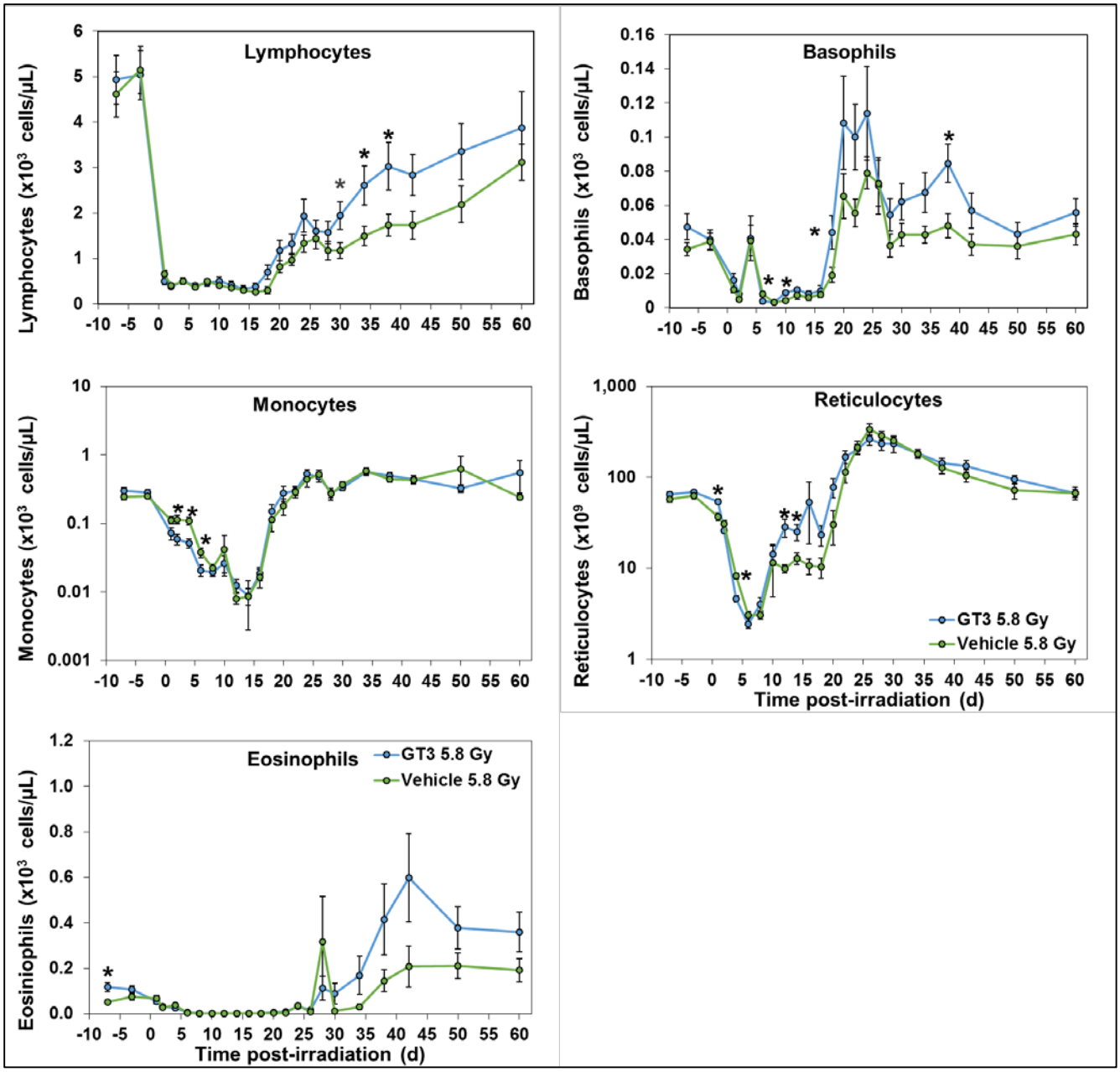


Figure 3: **GT3 induced changes in NHP lymphocytes, monocytes, eosinophils, basophils and reticulocytes.** Sixteen NHPs were injected with GT3 (37.5 mg/kg) and an additional 16 NHPs were injected with vehicle 24 h before exposure to 5.8 Gy (0.6 Gy/min) ^{60}Co γ-radiation. Blood was collected at various time points and cells were counted using a Bayer Advia-120 cell counter. The data for each time point is presented as the mean ± standard error for each treatment group.The data for each time point is presented as the mean ± standard error for each treatment group. '*' indicates a significant difference between GT3-treated group and vehicle-treated group, when equal variance between groups was assumed. ($p \leq 0.05$).

Appendix D. Blood Chemistry Parameters (5.8 Gy)

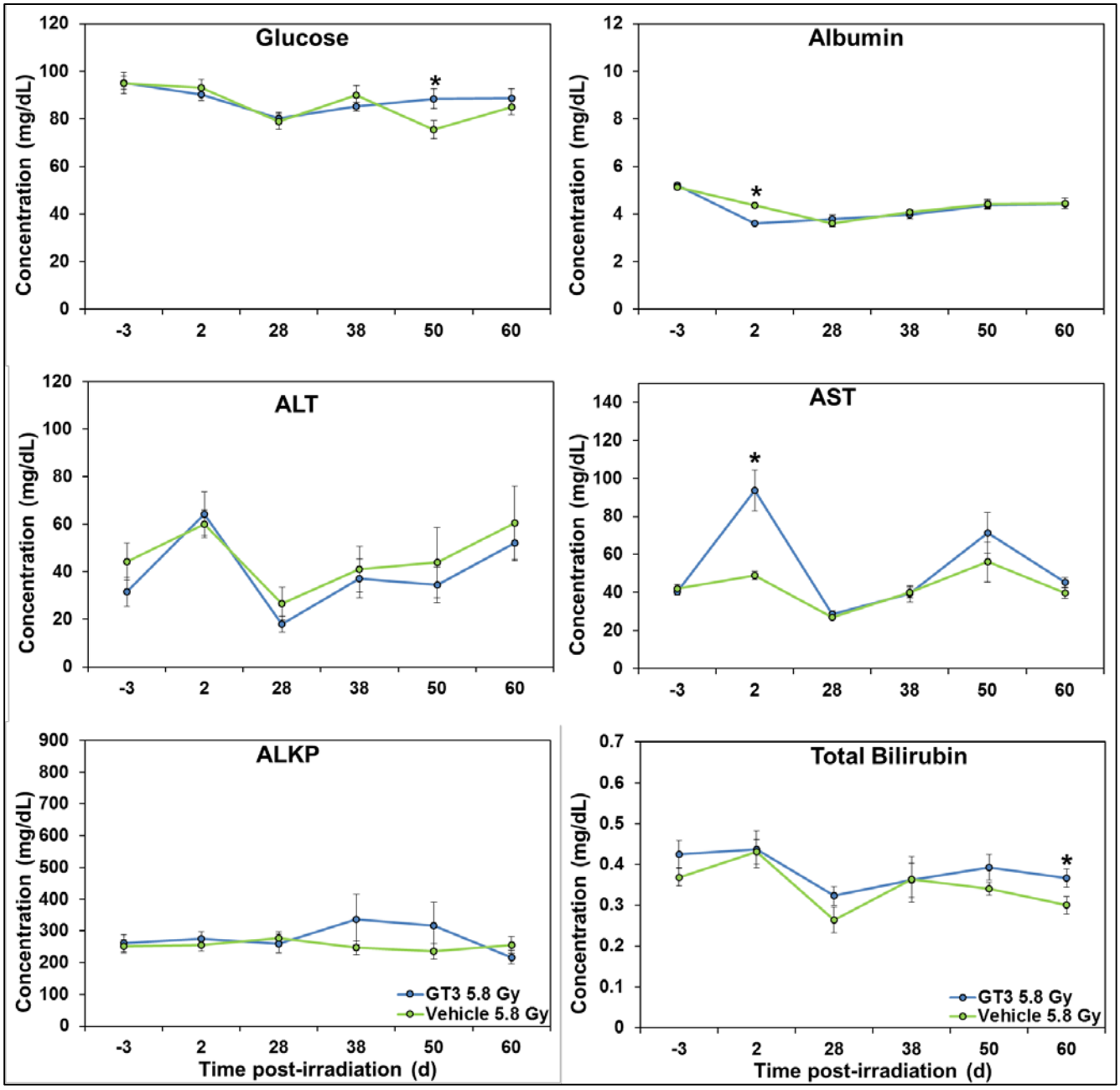


Figure 1: **GT3 induced changes in NHP glucose, albumin, ALT, AST, ALKP, and total bilirubin.** Sixteen NHPs were injected with GT3 (37.5 mg/kg) and an additional 16 NHPs were injected with vehicle 24 h before exposure to 5.8 Gy (0.6 Gy/min) ^{60}Co γ-radiation. Blood was collected at various time points and cells were counted using a Ortho Diagnostics Vitros 350 Chemistry System. The data for each time point is presented as the mean ± standard error for each treatment group. *' indicates a significant difference between GT3-treated group and vehicle-treated group, when equal variance between groups was assumed. ($p \leq 0.05$).

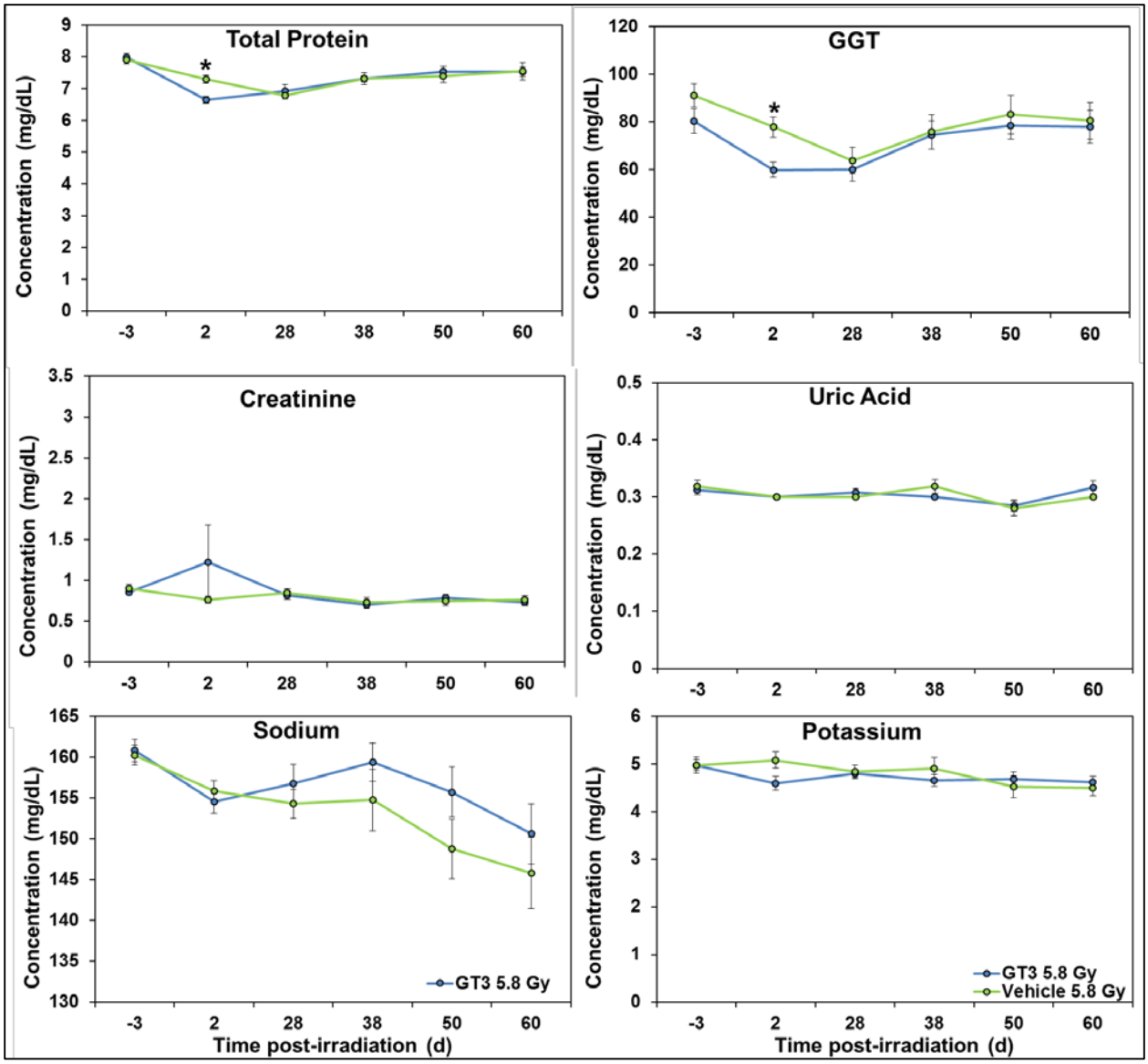


Figure 2: **GT3 induced changes in NHP total protein, GGT, creatinine, uric acid, sodium and potassium.** Sixteen NHPs were injected with GT3 (37.5 mg/kg) and an additional 16 NHPs were injected with vehicle 24 h before exposure to 5.8 Gy (0.6 Gy/min) ^{60}Co γ-radiation. Blood was collected at various time points and cells were counted using a Ortho Diagnostics Vitros 350 Chemistry System. The data for each time point is presented as the mean ± standard error for each treatment group. *' indicates a significant difference between GT3-treated group and vehicle-treated group, when equal variance between groups was assumed. ($p \leq 0.05$).

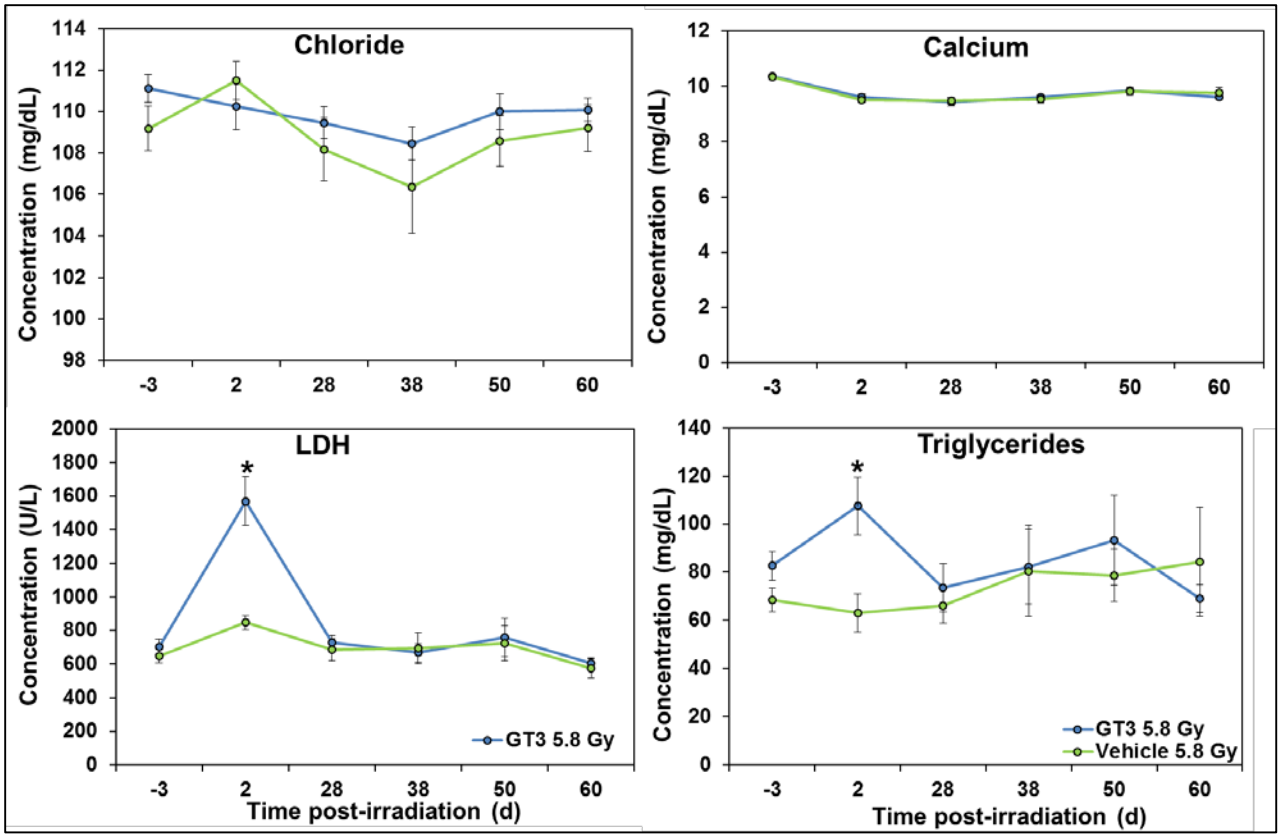


Figure 3: **GT3 induced changes in NHP chloride, calcium, LDH and triglycerides.** Sixteen NHPs were injected with GT3 (37.5 mg/kg) and an additional 16 NHPs were injected with vehicle 24 h before exposure to 5.8 Gy (0.6 Gy/min) ^{60}Co γ-radiation. Blood was collected at various time points and cells were counted using a Ortho Diagnostics Vitros 350 Chemistry System. The data for each time point is presented as the mean ± standard error for each treatment group. '*' indicates a significant difference between GT3-treated group and vehicle-treated group, when equal variance between groups was assumed. ($p \leq 0.05$).

Appendix E: Comparison of Various Cytokines (5.8 Gy)

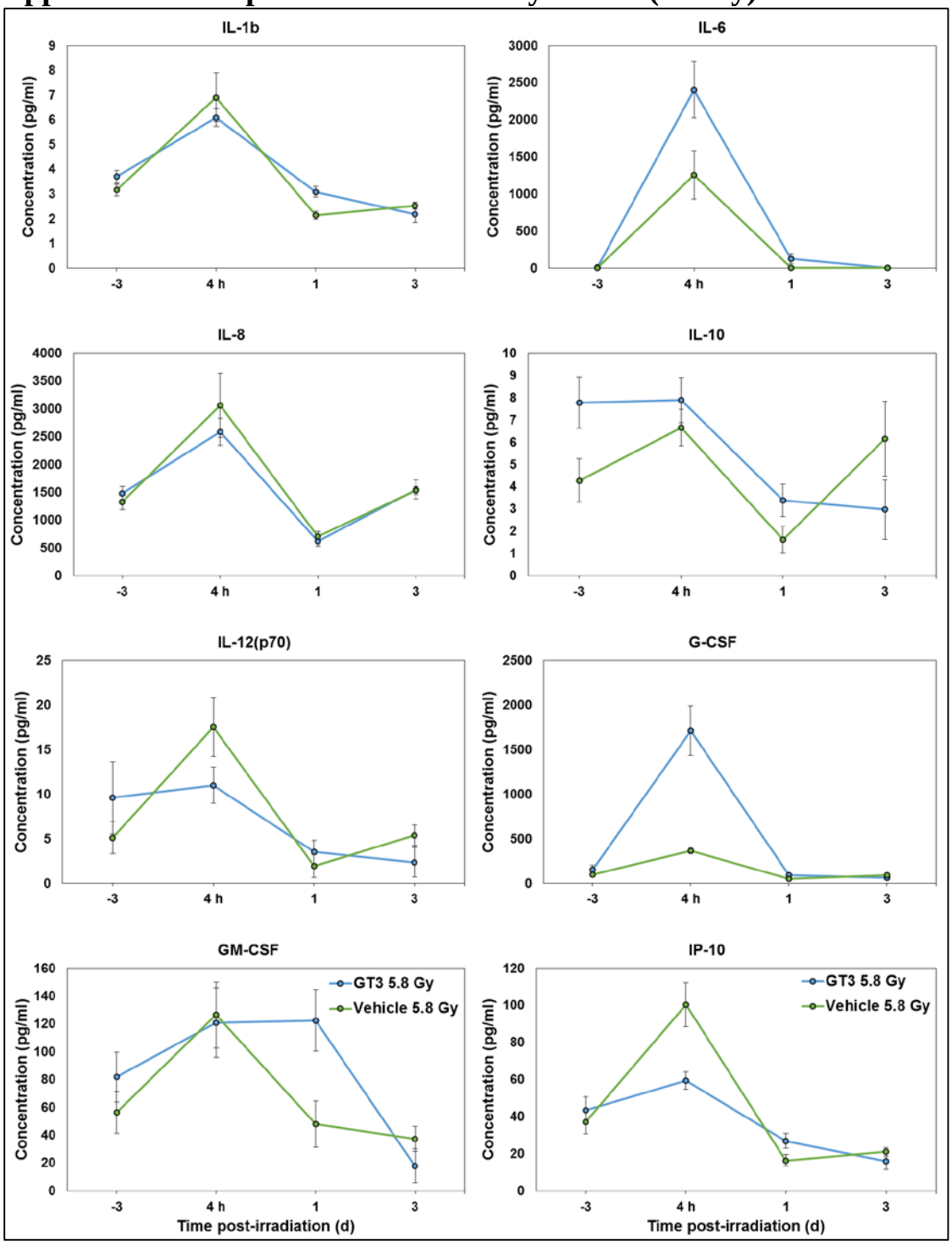


Figure 1: **Effects of GT3 administration on cytokine induction in GT3 treated NHPs at various time points.** Sixteen NHPs were administered GT3 (37.5 mg/kg) sc and an additional 16 NHPs were administered vehicle 24 h before blood collection. Blood samples were collected at various time points in relation to drug administration. Serum samples were analyzed by multiplex Luminex for cytokines. The data for each time point is presented as the mean ± standard error.

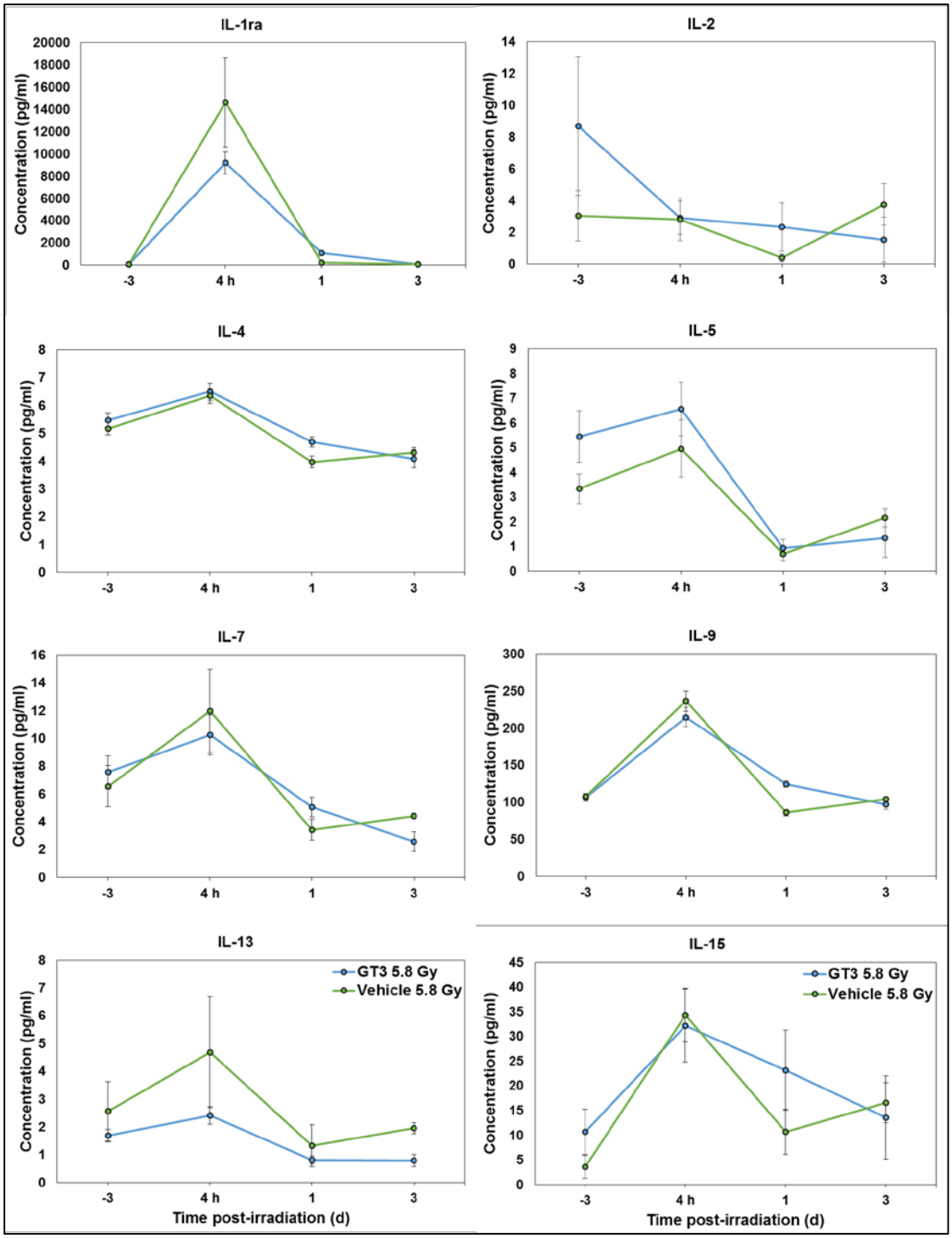


Figure 2: **Effects of GT3 administration on cytokine induction in GT3 treated NHPs at various time points.** Sixteen NHPs were administered GT3 (37.5 mg/kg) sc and an additional 16 NHPs were administered vehicle 24 h before blood collection. Blood samples were collected at various time points in relation to drug administration. Serum samples were analyzed by multiplex Luminex for cytokines. The data for each time point is presented as the mean ± standard error.

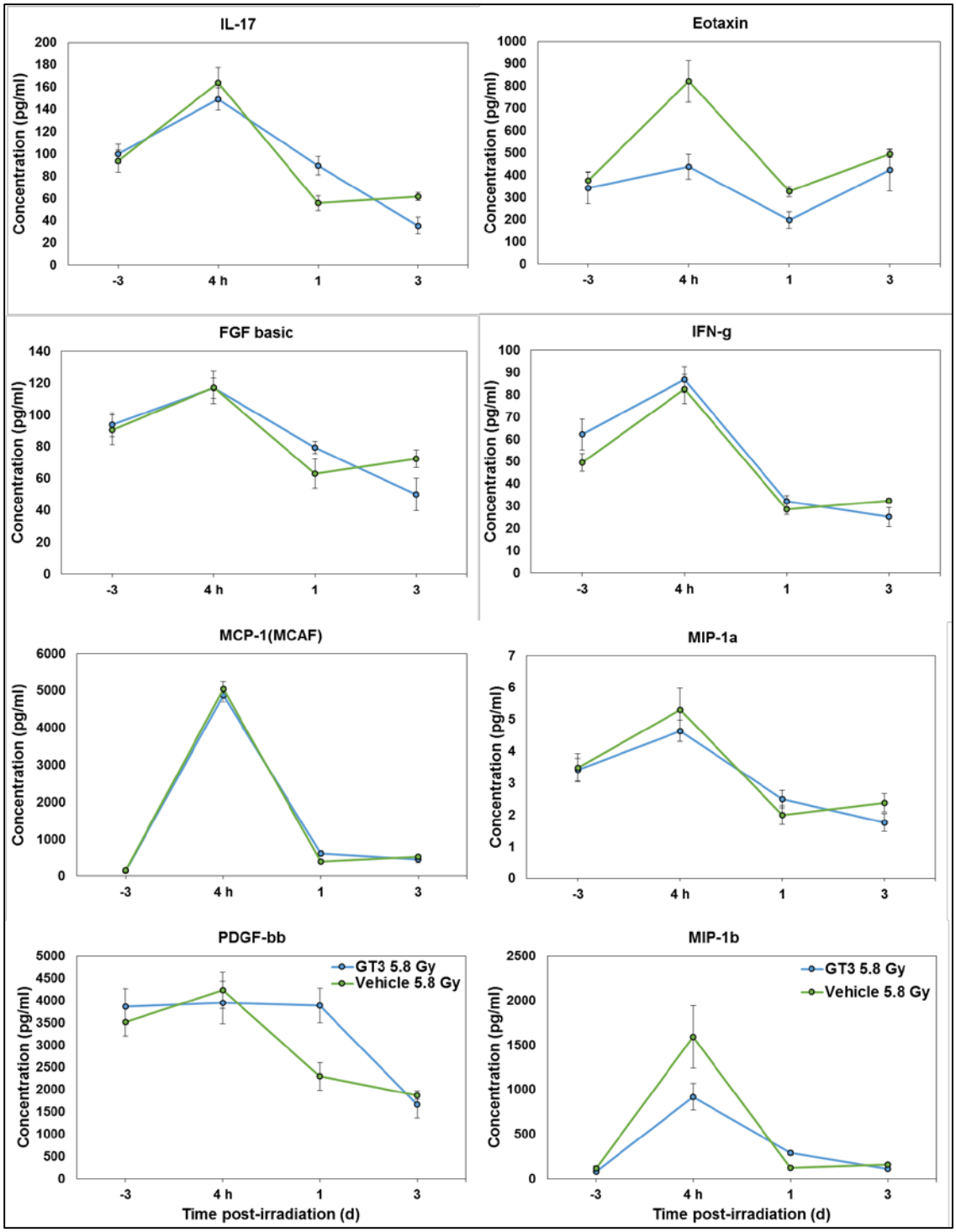


Figure 3: **Effects of GT3 administration on cytokine induction in GT3 treated NHPs at various time points.** Sixteen NHPs were administered GT3 (37.5 mg/kg) sc and an additional 16 NHPs were administered vehicle 24 h before blood collection. Blood samples were collected at various time points in relation to drug administration. Serum samples were analyzed by multiplex Luminex for cytokines. The data for each time point is presented as the mean ± standard error.

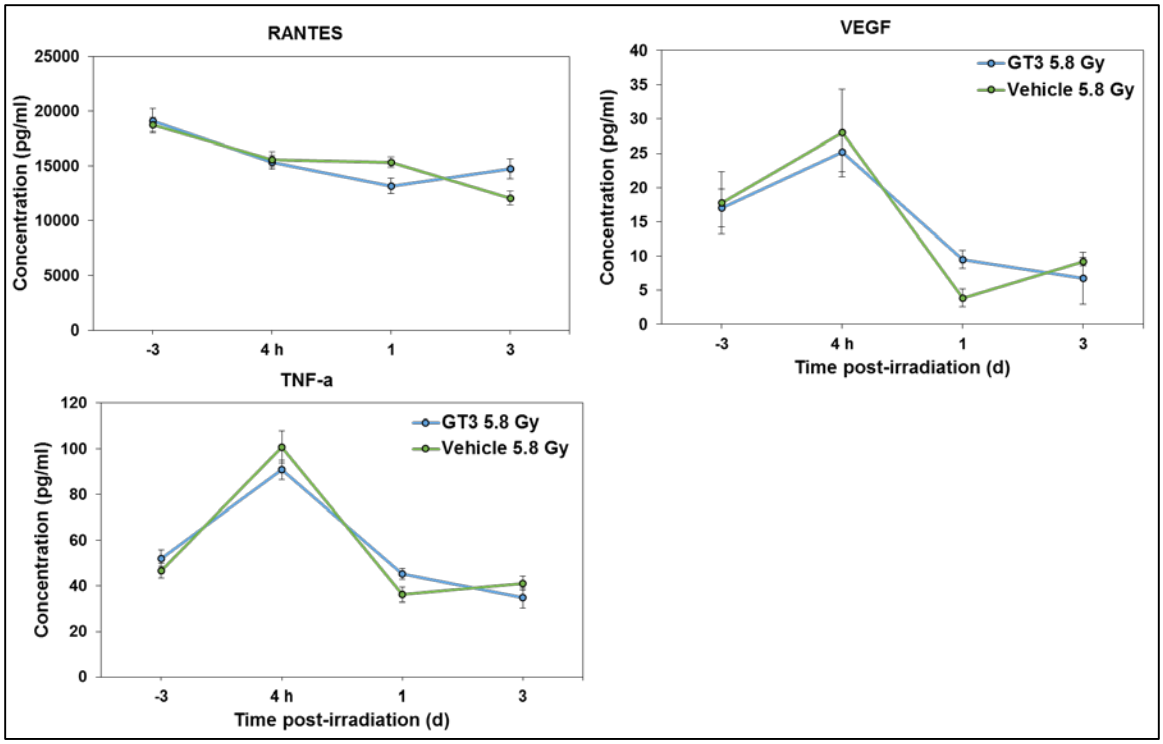


Figure 4: **Effects of GT3 administration on cytokine induction in GT3 treated NHPs at various time points.** Sixteen NHPs were administered GT3 (37.5 mg/kg) sc and an additional 16 NHPs were administered vehicle 24 h before blood collection. Blood samples were collected at various time points in relation to drug administration. Serum samples were analyzed by multiplex Luminex for cytokines. The data for each time point is presented as the mean ± standard error.

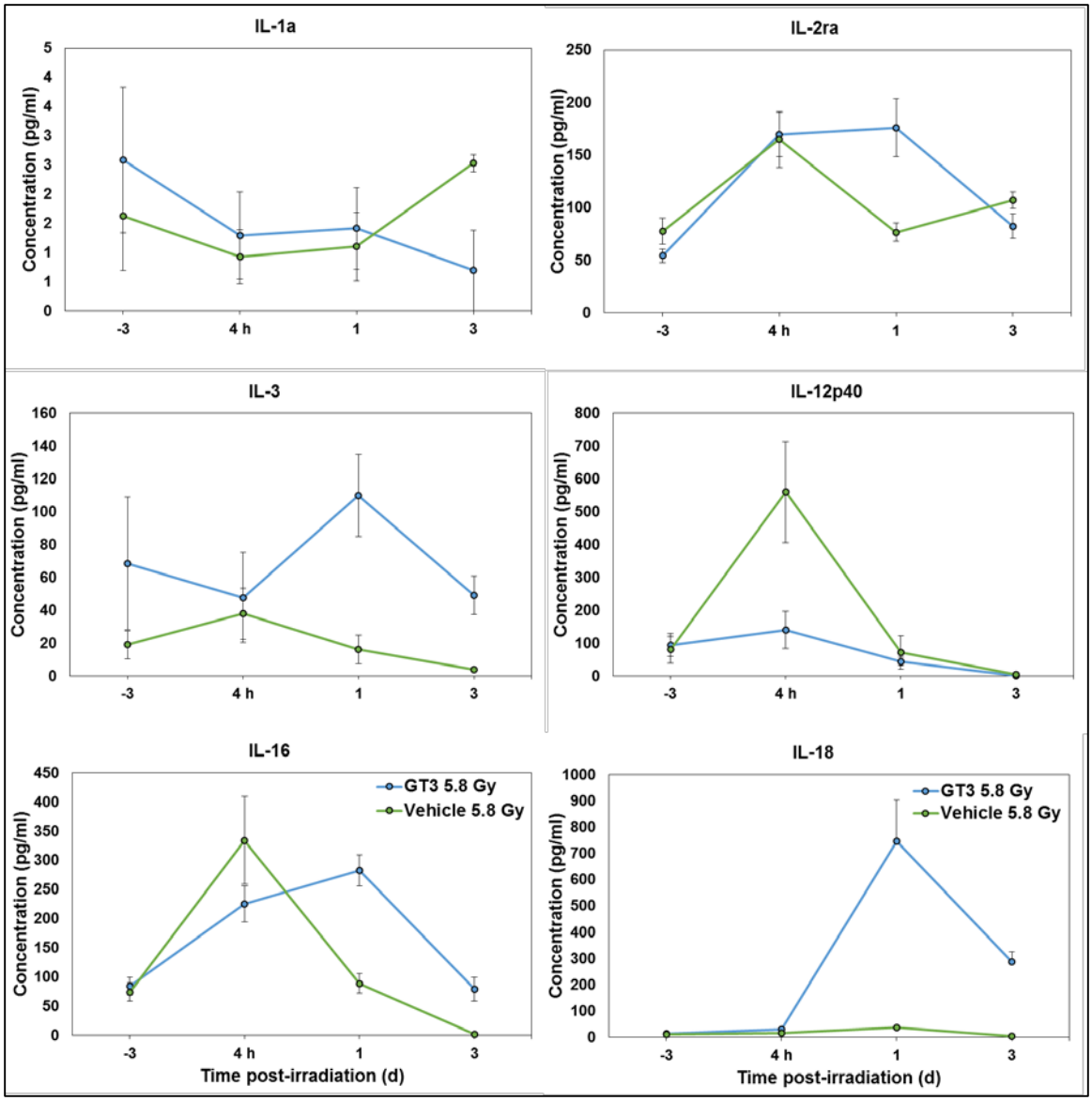


Figure 5: **Effects of GT3 administration on cytokine induction in GT3 treated NHPs at various time points.** Sixteen NHPs were administered GT3 (37.5 mg/kg) sc and an additional 16 NHPs were administered vehicle 24 h before blood collection. Blood samples were collected at various time points in relation to drug administration. Serum samples were analyzed by multiplex Luminex for cytokines. The data for each time point is presented as the mean ± standard error.

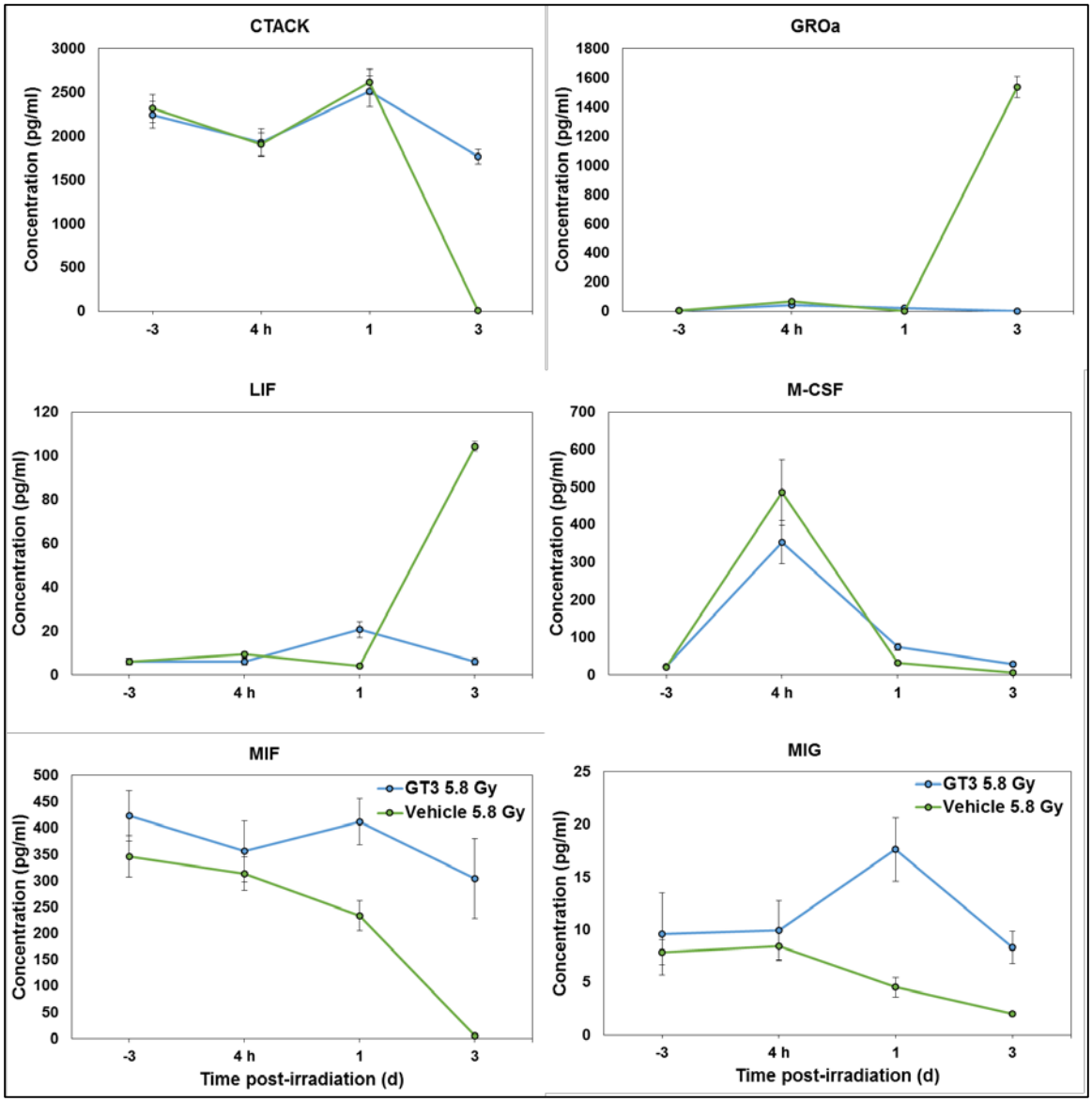


Figure 6: **Effects of GT3 administration on cytokine induction in GT3 treated NHPs at various time points.** Sixteen NHPs were administered GT3 (37.5 mg/kg) sc and an additional 16 NHPs were administered vehicle 24 h before blood collection. Blood samples were collected at various time points in relation to drug administration. Serum samples were analyzed by multiplex Luminex for cytokines. The data for each time point is presented as the mean ± standard error.

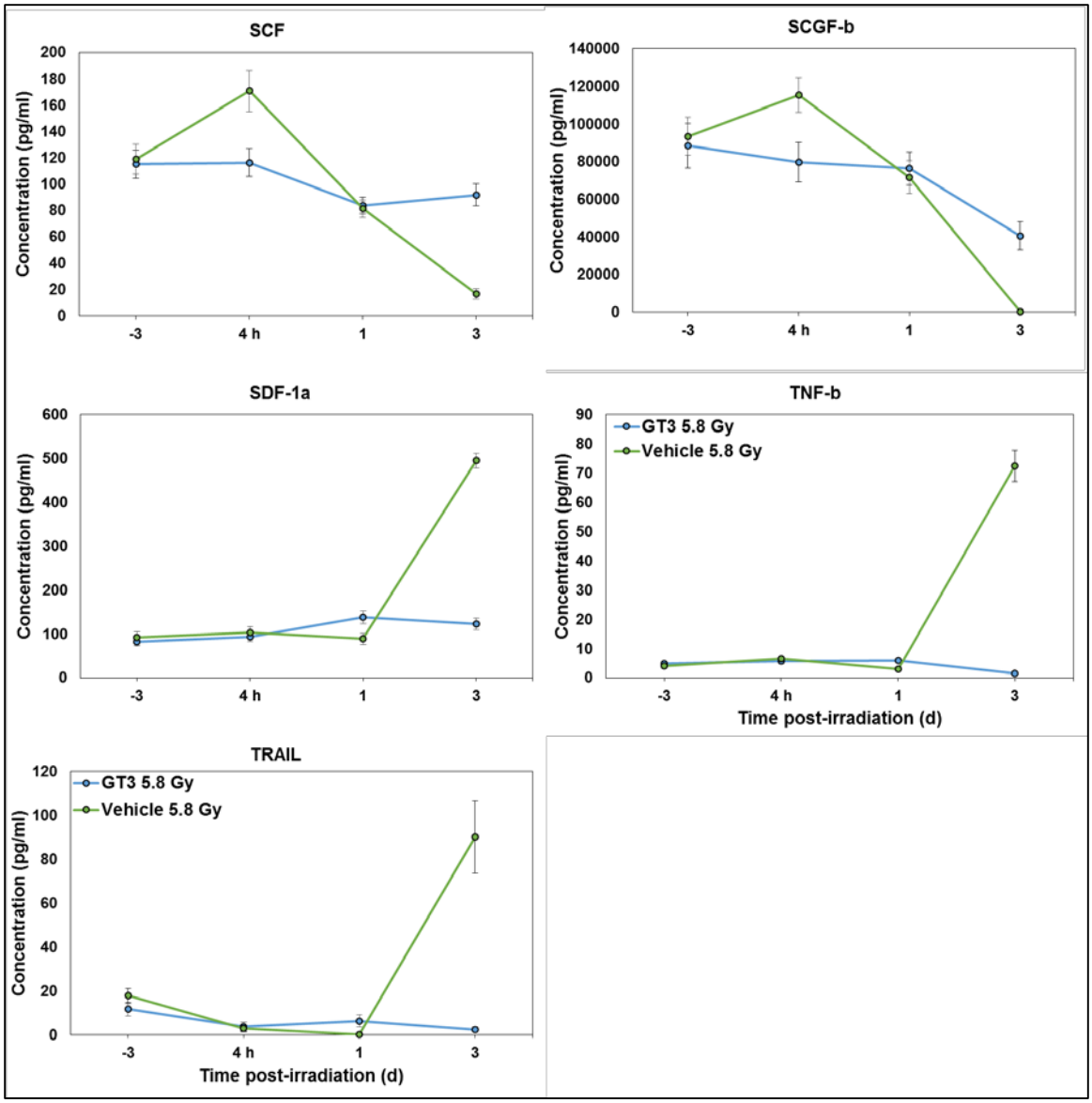


Figure 7: **Effects of GT3 administration on cytokine induction in GT3 treated NHPs at various time points.** Sixteen NHPs were administered GT3 (37.5 mg/kg) sc and an additional 16 NHPs were administered vehicle 24 h before blood collection. Blood samples were collected at various time points in relation to drug administration. Serum samples were analyzed by multiplex Luminex for cytokines. The data for each time point is presented as the mean ± standard error.

Appendix F: Vital Signs

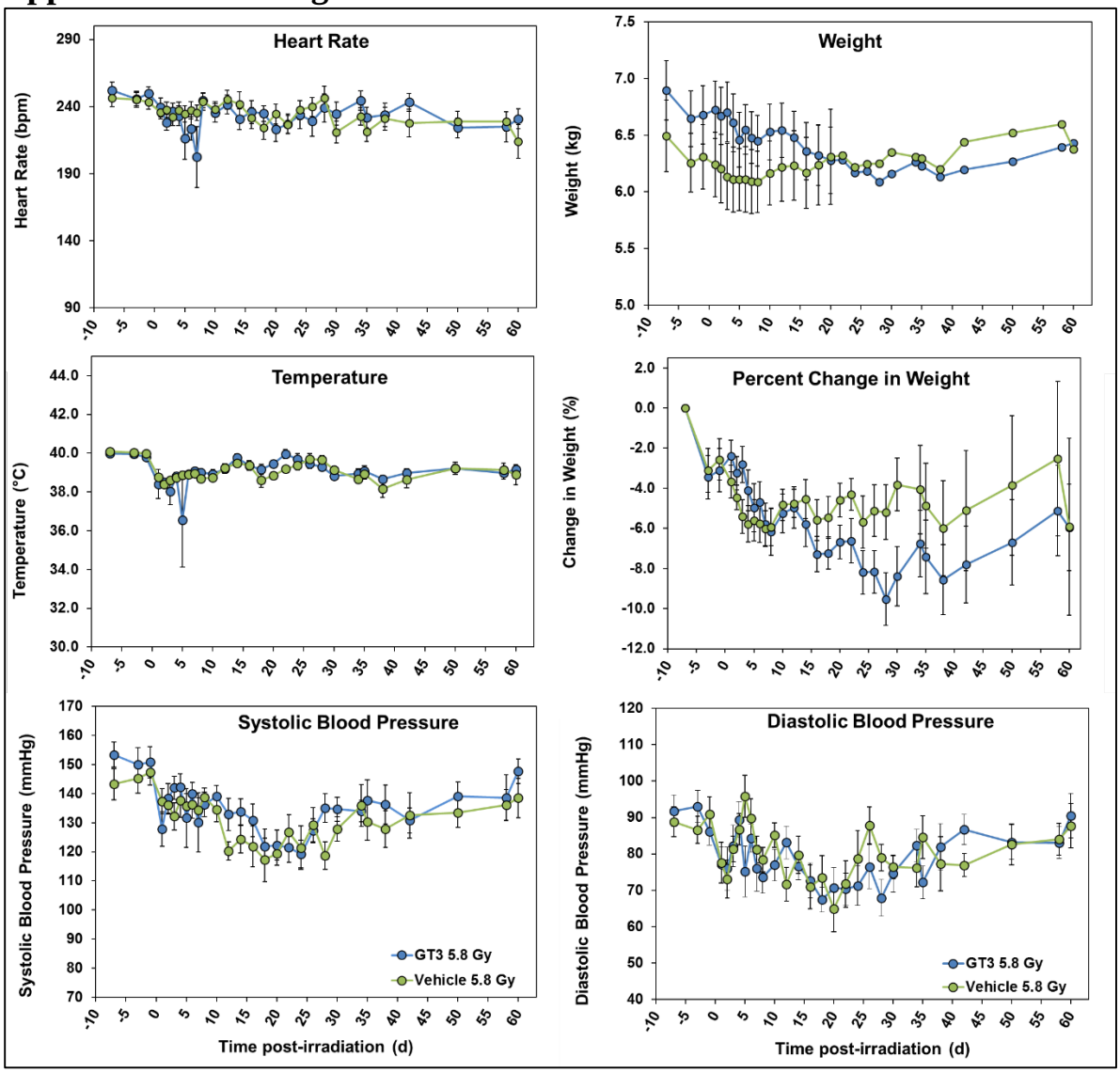


Figure 1: **GT3 induced changes in various NHP vital signs.** Sixteen NHPs were injected GT3 (37.5 mg/kg) and 16 NHPs were injected vehicle 24 h exposure to 5.8 Gy (0.6 Gy/min) ^{60}Co γ-radiation. Vital signs were collected at various time points throughout The data for each time point is presented as the mean ± standard error for each treatment group.

Appendix G: Collaborator Data

Table 1. CFU Assay Results (Combined)

NHP #	Total cells /10*7	$CD45^+$ cells %	$CD45^+CD34^+$ cells %	CFU-GM /10*5 cells	BFU-E /10*5 cells
RA1413					
RA1272					
RA1352					
RA9717	2.75	88.719	23.734	56.5	11
RA0946	2.42	93.897	29.737	29.5	5.5
RA1889	1.95	95.075	4.445	46	9.5
RA1773	2.68	99.357	4.155	51.5	9.5
RA1763	2.35	98.423	9.334	31	14
RA1164	3.08	96.031	6.025	38	6.5
RA0729					
RA1360	2.68	93.297	6.367	75	11.5
Average (Vehicle)	2.56	94.97	11.97	46.79	9.64
RA0652					
RA1307	5.72	94.013	43.471	36	10.5
RA0951	6.27	90.329	7.695	32.5	15
RA1917					
RA0520	3.96	96.193	6.696	76	20.5
RA1985	3.65	97.76	7.546	63.5	11.5
RA1558					
RA9577	4.21	92.893	6.401	65.5	10.5
RA1291	4.69	97.995	9.346	54.5	8
RA0283	6.35	87.568	5.127	46.5	13.5
RA1330	2.48	96.38	6.379	41.5	13
Average (GT3)	4.67	94.14	11.58	52.00	12.81

Flow Cytometry Analysis

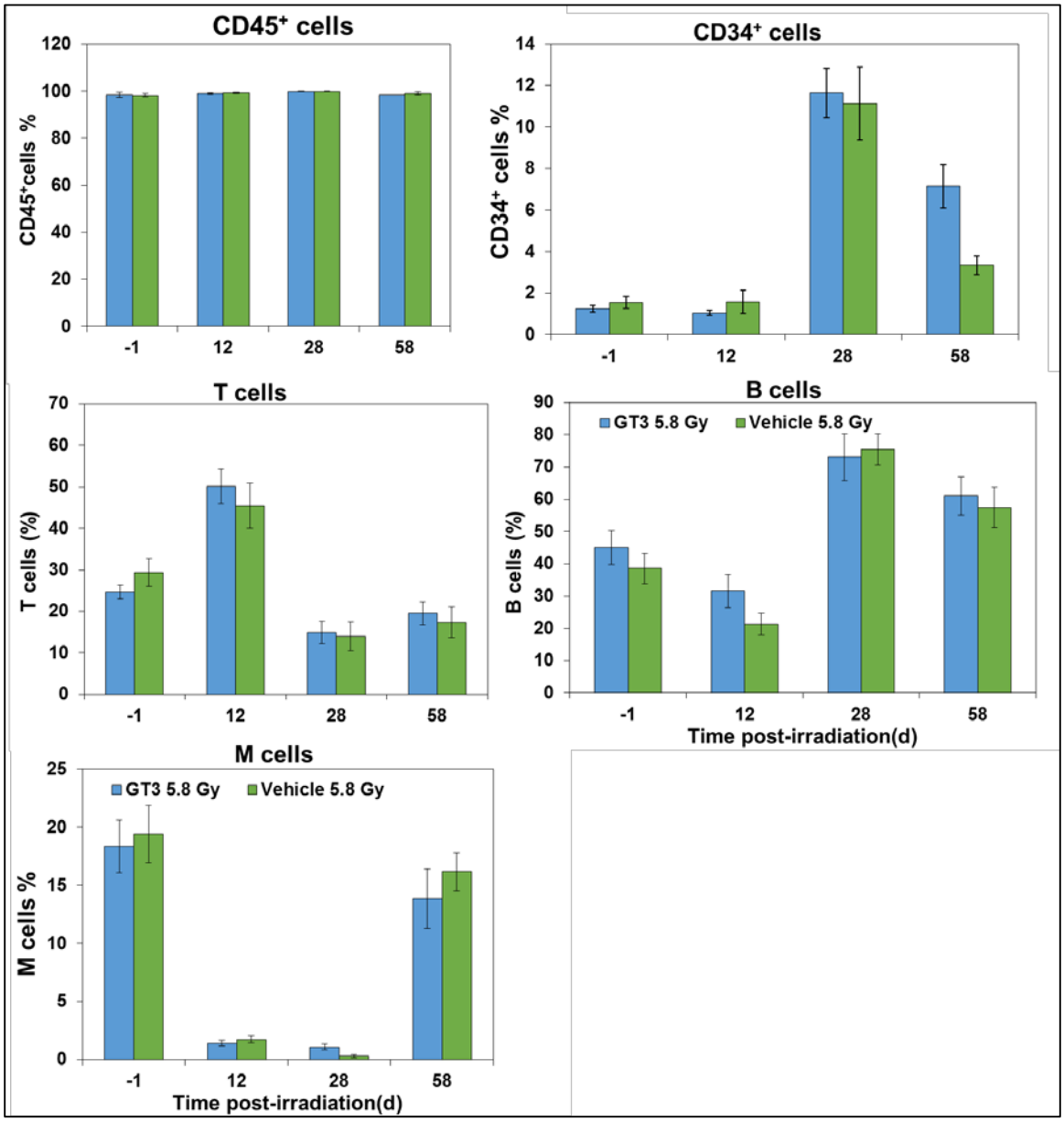


Figure 1: **GT3 induced changes in the distributions of various populations of hematopoietic cells in NHP blood**. Sixteen NHPs were injected GT3 (37.5 mg/kg) and an additional 16 NHPs were injected vehicle 24 h before exposure to 5.8 Gy (0.6 Gy/min) ^{60}Co γ-radiation . Blood was collected at various time points and later shipped to our collaborators at UAMS.

D-Dimer Analysis

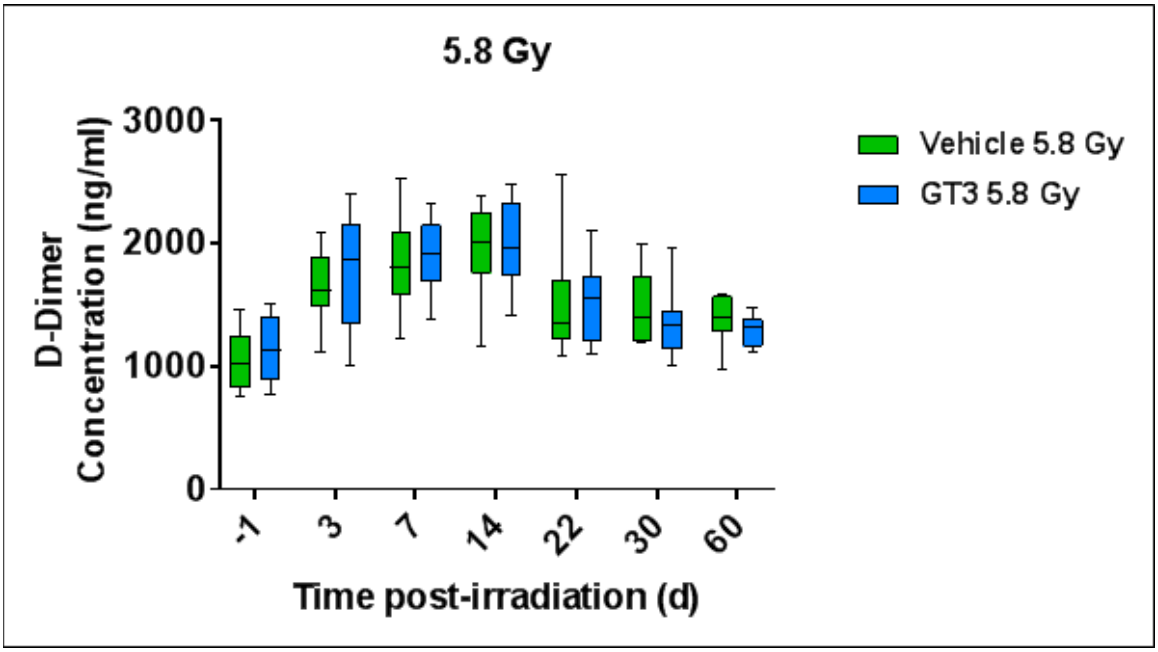


Figure 2: **GT3 induced changes in NHP plasma D-Dimer concentration.** Sixteen NHPs were injected GT3 (37.5 mg/kg) and an additional 16 NHPs were injected vehicle 24 h before exposure to 5.8 Gy (0.6 Gy/min) ^{60}Co γ-radiation.Plasma was collected at various time points and then later shipped to our collaborators at UAMS.

Thrombomodulin (TM) Analysis

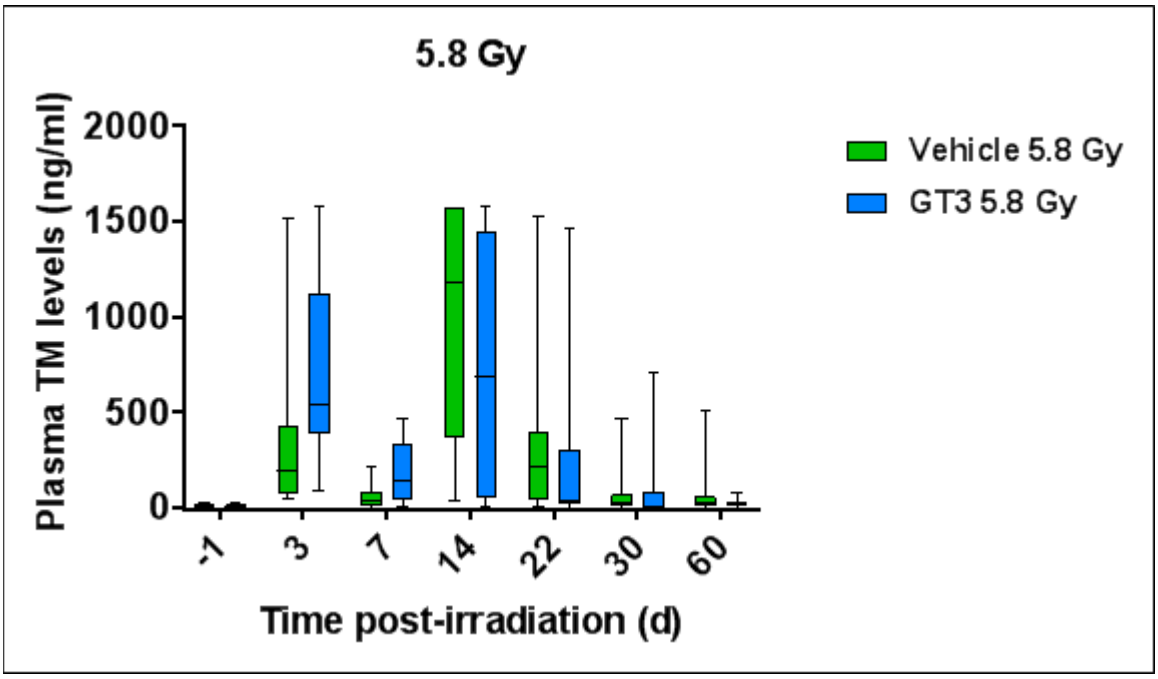


Figure 3: **GT3 induced changes in NHP plasma TM levels.** Sixteen NHPs were injected GT3 (37.5 mg/kg) and an additional 16 NHPs were injected vehicle 24 h before exposure to 5.8 Gy (0.6 Gy/min) ^{60}Co γ-radiation.Plasma was collected at various time points and then later shipped to our collaborators at UAMS.

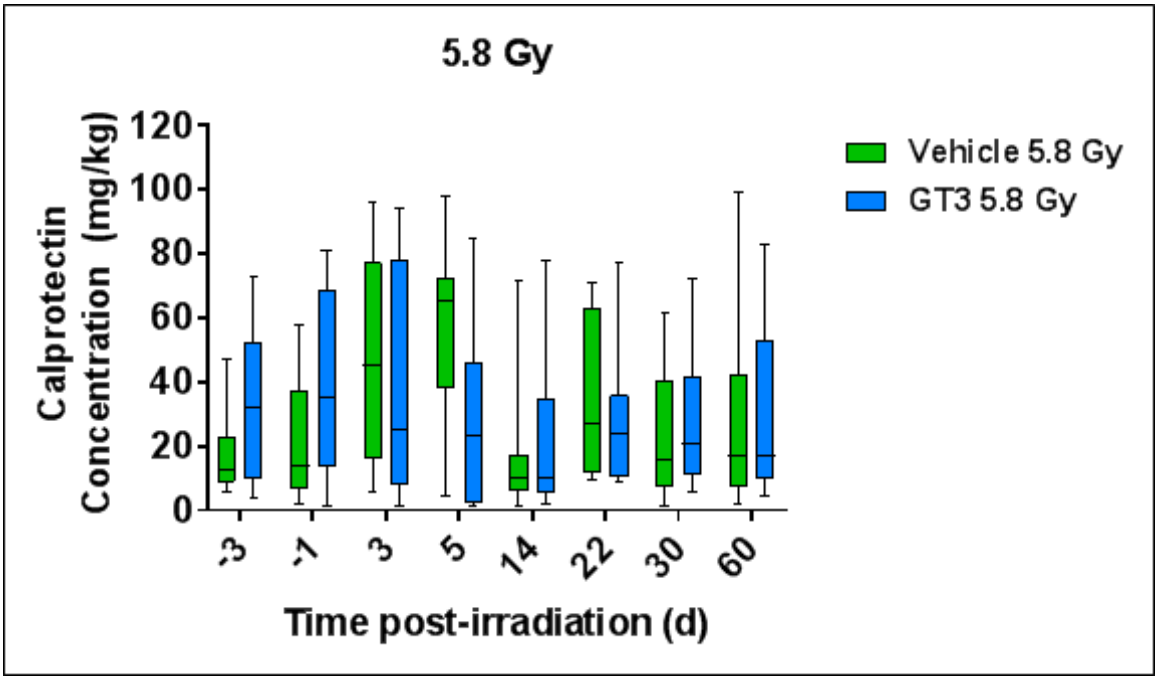


Figure 4: **GT3 induced changes in NHP fecal calprotectin concentration.** Sixteen NHPs were injected GT3 (37.5 mg/kg) and another 16 NHPs were injected vehicle 24 h after exposure to 5.8 Gy (0.6 Gy/min) ^{60}Co γ-radiation. Fecal samples were collected at various time points and then later shipped to our collaborators at UAMS.

Citrulline Analysis

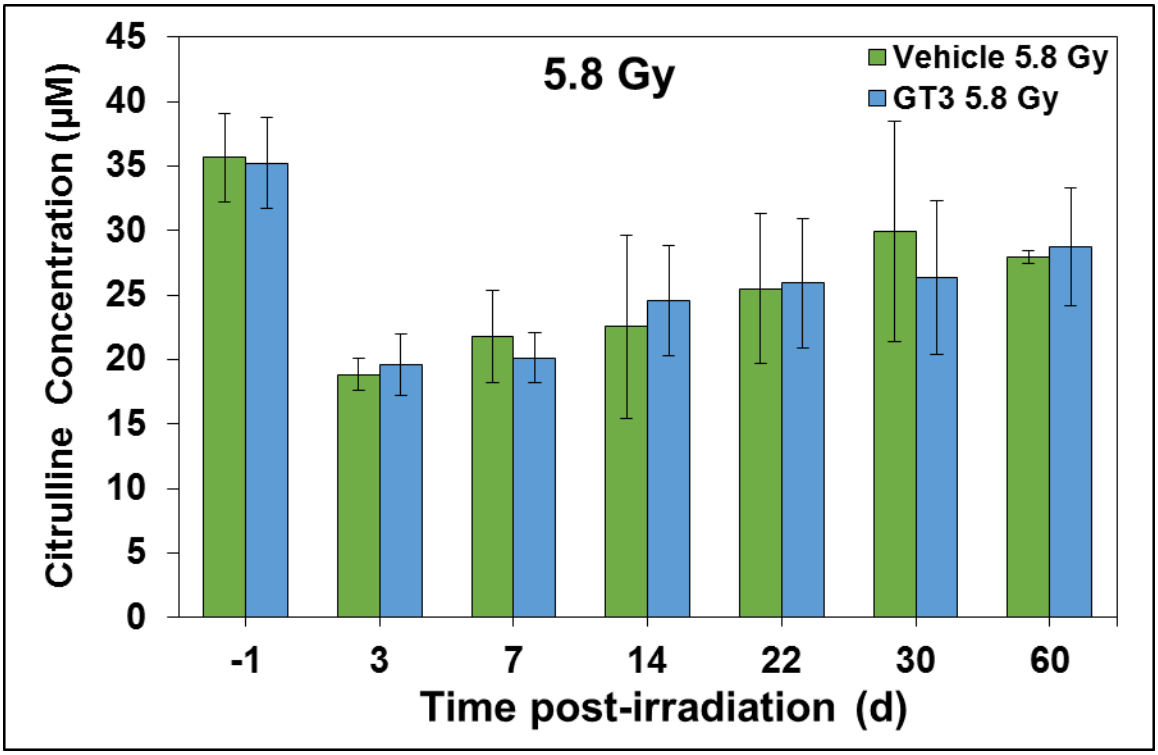


Figure 5: **GT3 induced changes in NHP plasma citrulline levels**. Sixteen NHPs were injected GT3 (37.5 mg/kg) and an additional 16 NHPs were injected vehicle 24 h before exposure to 5.8 Gy (0.6 Gy/min) ^{60}Co γ-radiation .Plasma was collected at various time points and then later shipped to our collaborators at UAMS

Micronuclei Analysis

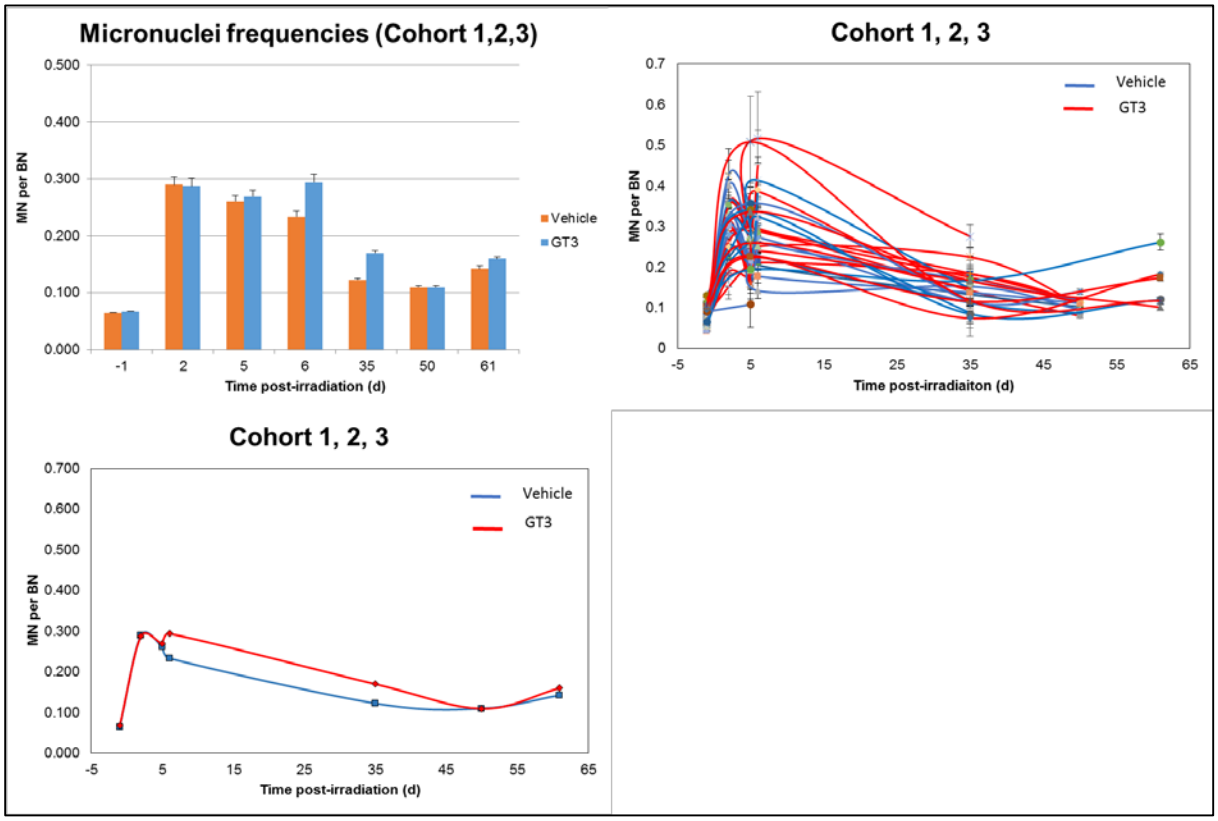


Figure 6: **GT3 induced effects on micronuclei per binucleated cells**. Sixteen NHPs were injected GT3 (37.5 mg/kg) and an additional 16 NHPs were injected vehicle 24 h before exposure to 5.8 Gy (0.6 Gy/min) ^{60}Co γ-radiation. Blood was collected at various time points and later shipped to our collaborators at Columbia University. The data for each time point is presented as the mean ± standard error for each treatment group.

Advanced Development of Gamma-tocotrienol as a Radiation Countermeasure

JW140032, Joint Warfighter Medical Research Program W81XWH-15-C-0117

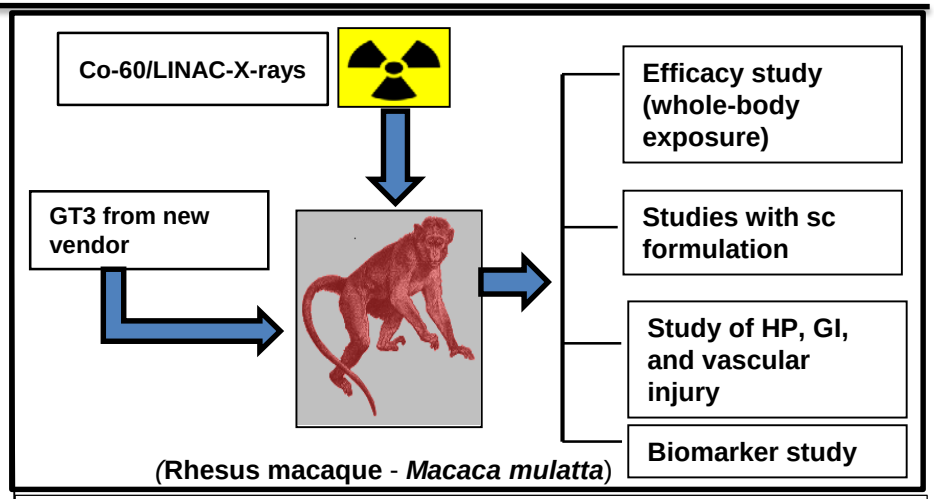
PI: Vijay K. Singh **Org:** AFRRI-HMJF/University of Arkansas for Medical Sciences **Award Amount:** $7,484,649

Study/Product Aim(s)

- Conduct efficacy study using whole-body ^{60}Co γ-radiation exposure in NHP model and identify effective formulation.
- Investigate the mechanism of action of GT3 for hematopoietic (HP), gastrointestinal (GI), and vascular injury in NHP model using partial-body and whole-body radiation exposures.
- Identify efficacy biomarkers for GT3 in un-irradiated and irradiated NHPs.

Approach

NHPs will be administered GT3, 24 h prior to ^{60}Co whole-body γ-radiation exposure and monitored for 60 days. Formulation of GT3 for sc administration will be tested to improve efficacy and reduce any side effects (injection site irritation for sc route). NHPs will be evaluated for hematopoietic (HP) recovery, citrulline, and cytokine levels. HP and GI recovery will be studied using partial-body and whole-body radiation exposure in NHP model. Efficacy biomarkers for GT3 will also be studied.



A pilot study demonstrated significantly improved HP recovery in single dose, GT3-treated irradiated NHPs without supportive care, comparable to that of published efficacy results of NHPs, treated with multiple doses of G-CSF and supportive care.

Timeline and Cost

Activities / CY	16	17	18	19
Evaluation of GT3 from New Vendor for PK and Skin Irritation				
Radioprotective Efficacy of GT3 against Two Different Doses of Radiation: Whole Body Exposure				
Study with Whole Body Radiation Exposure-Mechanistic				
Study with Partial Body Radiation Exposure				
Estimated Budget ($K)	**$1.9 mil**	**$1.9 mil**	**$2.0 mil**	**$2.0 mil**

Updated: 09/29/2016

Goals/Milestones

CY16 Goal – Test GT3 for PK/skin irritation

☑ Investigate GT3 for PK and skin irritation in NHPs

CY17 Goals – Efficacy study with optimal formulation

☐ Efficacy studies against ^{60}Co whole body γ-irradiation

CY18 Goal – Study mechanism of GT3 efficacy

☐ Study HP, GI, and vascular injury and accelerated recovery by GT3 in whole-body

CY19 Goal – Efficacy biomarker studies

☐ Study partial body irradiation for hematopoietic and gastrointestinal syndrome: Various time points.

Comments/Challenges/Issues/Concerns

None

Budget Expenditure to Date

Obligated Expenditure: $3,203,018.07

Actual Expenditure: $992,072.01